JOURNAL OF AGRICULTURAL RESEARCH

VOL. 38 WASHINGTON, D. C., JANUARY 15, 1929 No. 2

SYMBIOTIC FUNGI OF CEREAL SEEDS AND THEIR RELATION TO CEREAL PROTEINS [1]

By S. L. JODIDI
Bureau of Plant Industry
United States Department of Agriculture

and

JAROSLAV PEKLO [2]
Technical University of Prague, Czechoslovakia

MYCOLOGICAL INTRODUCTION

In 1913 the junior writer (*27*)[3] published his early observations that the so-called aleurone layer and other constituents of cereal and grass seeds are produced by symbiotic fungi. He maintained that the aleurone grains in the aleurone layer and in the embryo represent offshoots of their hyphae and arise from minute hyphal prominences in this hereditary symbiosis. While examining microtome sections of young endosperms of cereals and of some kinds of wild grasses he noticed that the peripheral layers of the endosperm were also penetrated by many strands of fungal hyphae. He found that in older seeds most of the masses were greatly swollen and mucilaginous and constituted a great part if not the whole of the gluten contents of the endosperm.

The first attempts to isolate the symbiotic fungi were made in London with young seeds of English ryegrass (*Lolium perenne*), because this plant contains the symbiotic fungus not only in the aleurone layer and in the endosperm but also between the aleurone layer and the seed coat, where it forms a distinct mycelium connected with the specific contents of the aleurone cells, its product. From young fruits of selected plants, which showed no trace of a smut on their surfaces or any other infection in their tissues when examined microscopically, a part of the intercellular fungal layer was aseptically separated and transferred to urea gelatin. After hundreds of unsuccessful trials, in some cases the organism grew on the gelatin and produced small mycelia, the hyphae of which were identical with those forming the layer. Its development was checked step by step with a microscope. It was propagated and showed the mycelial characters, sporidia, nuclei, and black, thick-walled chlamydospores characteristic of a smut.

Attempts were then made in Prague, Czechoslovakia, on a very large scale to isolate the symbiotic fungi from young seeds of cereals. These attempts were unsuccessful for some years, or until a special method of isolation was used. In order to make sure that the fungal components belonged to symbiotic smuts, the only material used

[1] Received for publication Mar. 10, 1928; issued February, 1929.
[2] Doctor Peklo, phytopathologist, acted as voluntary collaborator in this investigation.
[3] Reference is made by number (italic) to "Literature cited," p. 90.

was that which showed no trace of contamination with any parasitic smuts (Tilletia and *Ustilago tritici* in winter and spring wheat, and *U. hordei* and *U. nuda*) and Helminthosporium in spring barley. The junior writer was in charge of large fields containing material that was destined for propagating the certified seed of the latest breeding rank. The material used was thoroughly disinfected with hot water against *U. tritici* and *U. hordei*, respectively, and with chemicals against the other fungi. Not a single plant showed a trace of any of the above-mentioned parasites on the surface or in the interior of the seeds, as was demonstrated by means of cross sections inspected at a high magnification before they were prepared for isolation trials.

Only the use of native proteins of different cereals in nutrient solutions was successful for the culture of the fungi. For this purpose, 130 gm. of young seeds of the above-mentioned cereals, respectively, were crushed, and the ground mass was extracted with 1,000 c. c. of distilled water. This juice was then sterilized with a Chamberland filter adjusted into a metal bomb of the original Chamberland apparatus. Air pressure of about 2 atmospheres was usually found sufficient. Only fresh filtrate was used. The best method was to take as small pieces as possible of aleurone layers (a half millimeter quadrate was sufficient) without any trace of adjacent tissues from coat or endosperm. Aleurone layers just after having been established, with only very fine granulation on the surfaces of symbiotic hyphae and extremely thin walls, gave the best results. Hanging drops or small quantities of the above-mentioned nutrient solution in small Erlenmeyer flasks were used.

After the incubation period had elapsed and the beginning of growth from the pieces was observed, these parts of tissues were taken from the nutrient solution, stained with dilute Löffler's methylene blue, and examined with the oil-immersion objective (1/12) and compensating ocular 8. The pieces of aleurone layers were not contaminated with any parasitic smuts or other parasitic fungi in a single case. However, great changes in the fungous cell contents of the aleurone layers were noticed. The aleurone hyphae had disintegrated or swollen in their course and at their ends into egg-shaped bodies which gave rise to yeastlike cells. They propagated themselves very profusely. When transferred to wort agar and examined in vivo they showed the presence of large nuclei with the structure characteristic of smuts. Somtimes they copulated as sporidia of the fungi under consideration do, and frequently they produced short or longer filaments with very peculiar starlike ramifications. When compared with the species and strains of parasitic smuts in the junior writer's mycological collection, i. e., with Tilletia, *Ustilago tritici*, *U. hordei*, *U. nuda*, *U. avenae*, *U. maidis*, and *Urocystis occulta*, as well as with smuts from other collections, they showed a very close morphological similarity, although they were not entirely identical with any of them; and when inoculated into sterilized seeds or plants of different cereals they failed to produce any known symptom of parasitism. They must be considered as smuts, but as a new type of smut different from any hitherto known, and they must also be considered symbiotic smuts.

The fungi discussed in this paper were isolated from a soft winter wheat, three kinds of spring wheat, three kinds of spring barley, and

one kind of winter rye, all of which were grown in Czechoslovakia. Moreover, some kinds of Indian spring barleys were compared, with the same result. Scores of strains have been isolated. At least 50 of these show a difference worth mentioning when cultivated after being purified in the usual mycological manner, and new strains still appear in the cultures. It has been observed that when these fungi are cultivated in liquid media in 1-liter flasks, and on a large scale in about 100 flasks, it is easier to detect new strains which are sometimes also of a physiological interest than when they are cultivated in slender test tubes. With regard to the question whether the isolated microorganisms belonged to the mycogenic parts of the cereal seeds, it should be recalled that from such a surprisingly large number of isolation trials only the same type of smuts was obtained. After the first difficulties in isolating had been overcome, nearly every series of isolations was successful. Moreover, a very considerable number of successful isolations in the same series must be taken into account. Thus, as a record number there can be registered fully 50 per cent of similar isolated strains from a field of the same variety of wheat. All this shows a very large, if not a constant, occurrence of these organisms in the tissues of cereal seeds and excludes the possibility that they are parasitic smuts. There must be present in the tissues of the small parts of aleurone layers employed numerous germs of organisms capable of propagation.

MYCOLOGICAL CONSIDERATIONS

Freeman (*3*, *4*, *5*), in a series of papers devoted to the cytological investigation of the symbiosis of *Lolium temulentum*, suggested that many facts concerning the morphology of the fungus in question and its occurrence in the tissues of its hosts indicated its possible affinity with the Ustilagineae. McLennan (*19*) carried on similar studies on *L. perenne*. Through a study of the fungus of *L. temulentum*, Peklo (*27*) found in 1913 that some hyphae penetrate from the fungus layer into the cells of the aleurone layer. In subsequent years he observed that the penetration occurs constantly and that the hyphae fill the lumina of these cells, turning around repeatedly and forming a fine network. It was but natural that the observations were extended to the aleurone layer and the subjacent tissues of cereals by means of the usual microtome and staining methods.

Young seeds of cereals (Triticum, Hordeum, Secale), especially of one kind of spring wheat, fixed and embedded in paraffin, proved very instructive. By means of sections it was possible to demonstrate that the contents of the aleurone cells consist of fungal hyphae and that the so-called aleurone grains represent their products. In the endosperm of *Triticum sativum*, Guilliermond found grains of proteins which could be stained "exactment comme les grains de proteine do l'aleuron." Fungal hyphae were observed by the junior writer in the same tissue in both barley and wheat.

At that time hundreds of unsuccessful attempts were made to isolate the fungus from the cereals. However, in 1923–24 a very good opportunity was afforded the junior writer to continue his research work on symbiosis in V. H. Blackman's laboratory of plant physiology and in S. B. Schryver's laboratory of biochemistry at the Imperial College of Science and Technology in London. There was found in

England's mild climate plenty of English ryegrass (*Lolium perenne*), which ripened very slowly and whose young fruits were very good material for repeated trials to isolate their fungus. English ryegrass at Prague, so far as the junior writer investigated it, did not contain a well-developed fungal layer and therefore was not suitable for starting isolations.

In London the isolations were made during October and November from plants growing in city and suburban parks. They were well developed and possibly belonged to different strains, but this point was not determined. About 25 per cent of the tested material contained well-developed intercellular fungous layers in every detail identical with the corresponding structures described for *Lolium temulentum* and *L. perenne* by previous investigators (*3*, *5*, *19*).

By far the most promising way of demonstrating the affinities of the fungus to its host seemed to be an inquiry into its chemical properties, especially its proteins. There are no phanerogams known except grasses and cereals that contain such specific proteins as gluten. As soon as the methods of cultivating the smut of *Lolium perenne* had been learned they were applied on a large scale. In accordance with Osborne's (*1*, *24*) methods of obtaining gliadin from wheat flour, after many preliminary trials the material was treated in London in the following manner: The fungus was cultivated in five Erlenmeyer flasks of 1 liter each containing 250 c. c. mineral solution of slight acidity with 1 per cent dextrose and 1 per cent peptone. Thus a protein of well-known composition containing no other nitrogen compound was offered the fungus. The well-developed mycelia were washed with great quantities of ether water in a large bottle. The fungus was killed, and the permeability of its cells increased. After thoroughly washing with distilled water, the fungus was collected and frozen in a freezing mixture. Dried with anhydrous ether, the mycelium was then treated with N/20 sodium hydroxide. Proteins extracted in this manner were precipitated with 2 N acetic acid. The precipitate was centrifuged, washed thoroughly with distilled water, centrifuged again, washed with 30 per cent ethyl alcohol, centrifuged again, and finally digested with 70 per cent ethyl alcohol at room temperature for 24 hours. A considerable quantity of the precipitate was dissolved. The alcoholic extract was concentrated in vacuo at a low temperature, the alcohol distilled off, and the remaining sirup transferred to a considerable quantity of ice water to which some sodium chloride was added. The precipitate, which was separated by means of a centrifuge, was soluble in 70 per cent alcohol and showed protein reactions. It apparently consisted largely of prolamin.

Some foundations for the physiological investigation of the aleurone symbiosis having thus been established, the junior writer availed himself of the opportunity to continue his research in the laboratories of the Bureau of Plant Industry, United States Department of Agriculture during 1927. Because the smut of *Lolium perenne* grows relatively slowly, and large quantities of it in a dry state were needed, pure cultures were grown on a larger scale in Peklo's laboratory of applied botany in Prague, and portions of dry substance from them were forwarded repeatedly in a perfect condition to Washington. The nutrient solution used for the cultures was made according to the

formula of A. Meyer and contained the following substances: 0.1 per cent KH_2PO_4, 0.1 per cent $MgSO_4$, 0.01 per cent $CaCl_2$, 0.01 per cent KCl, trace of NaCl and Fe_2Cl_6, 1 per cent dextrose (Merck's purissium), and 1 per cent peptone (Witte). The reaction was slightly acid.

Material was collected from the cultures from eight hundred and fifty 1-liter Erlenmeyer flasks, each containing 250 c. c. of the nutrient solution. After being tested for purity it was washed thoroughly with distilled water and ether water, dried with anhydrous ether on Büchner funnels, and sent to Washington. There were prepared from it 105.5 gm. of air-dry powder. This material was used for analysis.

For experiments with symbiotic smuts of cereals a strain (No. 13) isolated from Stocken winter wheat, a very soft kind of wheat, grown in Bohemia, was chosen. Pure cultures of it were grown in Washington from July 19 to September 16, 1927, in fifty-four 1-liter Erlenmeyer flasks, in 250 c. c. of nutrient solution of the following composition: Normal beef extract made alkaline with sodium hydroxide, 3 per cent dextrose (pure Difco), and 0.4 per cent asparagine (Merck). After the fungal content of each culture had been examined for purity it was filtered through Büchner funnels, washed with distilled water and then with ether water, and finally dried with anhydrous ether. It consisted of short filaments frequently united in a starlike fashion and of prevalent yeastlike "conidia." When removed from the filter papers the fungus yielded, in the first series, 16 gm. of air-dry powder and in the second 12.5 gm.

The symbiotic smut of barley that was used for analysis was strain No. 14a, isolated from Hanna barley from Bohemia. This kind of barley is very rich in carbohydrates and poor in proteins. It was cultivated at Washington in fifty-seven 1-liter flasks containing 250 c. c. of nutrient solution each, after A. Meyer's formula, plus 1 per cent dextrose (Difco) and 1 per cent peptone (Bacto-peptone). Its reaction, which was pH 5.9 at the time of inoculation, July 9, changed to pH 7.2 on September 17, when the analysis was begun. The cultures, which were tested for purity, consisted of long filaments with "conidia." After being filtered on Büchner funnels, washed with distilled and ether water, and dried with anhydrous ether, this material gave 9 gm. of air-dry powder. For comparison, the reactions of 116 flasks with the same fungus cultivated in A. Meyer's medium, plus 2 per cent dextrose and 1 per cent peptone were examined. At the time of inoculation, October 20, the pH was 5 and on November 1, when the experiment was completed, it was 6.9. This series yielded 40.7 gms. of air-dry powder. In order to accelerate the preparation of fungi for analysis and to avoid contamination with air-borne bacteria, a part of the material, taken from the flasks, was centrifuged, washed with ether water, and then put on Büchner funnels. The material collected and prepared by these methods was then used in the chemical work.

The junior writer is continuing the study of the fungi mentioned above and is working on further cytological details such as the origin of gluten from fungi and the occurrence of symbiotic fungi in seeds of plants other than cereals which contain aleurone grains.

BIOCHEMICAL CONSIDERATIONS

The biochemical work reported in this paper was done by the senior writer in active cooperation with the junior writer. It was prompted by the following considerations: From the numerous mycological observations made by Peklo (*27*, *29*, *30*) the conclusion seemed justified that the cells of the aleurone layer of English ryegrass, barley (*Hordeum vulgare*), and wheat (*Triticum vulgare*) are entered by symbiotic fungi that later penetrate also the cells of the endosperm, where, on dying, they leave the constituents present in their tissues. If such be the case it would be but natural to expect the symbiotic fungi to contain some if not all of the compounds that are known to occur in the kernels of the grasses mentioned. This suggested the employment of biochemical methods, especially in view of the fact that it seemed desirable, if not imperative, to verify the mycological observations made by methods quite different from those applied by the junior writer. Since the proteins are the most conspicuous constituents of cereal grains, the biochemical work presented here deals chiefly with these compounds. Moreover, because of the fact that alcohol-soluble proteins occur only in cereal grains and in no other plants and hence are very characteristic of cereals, special attention was paid to the occurrence and estimation of prolamins in the symbiotic fungi of the grasses under consideration.

From the investigations of Osborne and his associates it is known that alcohol-soluble proteins (prolamins) are present in the kernels of wheat (*24*, *25*, *26*), barley (*22*, *26*), rye (*Secale cereale*, *21*, *26*), maize (*Zea mays*, *23*, *26*), and oats (*Avena sativa*, *20*), while the work of Johns and Jones (*17*, *18*) and of Hoffman (*6*) showed that kafir (*Holcus sorghum*) and rice (*Oryza sativa*) also contain alcohol-soluble proteins. In this paper it is shown for the first time that English ryegrass contains a prolamin and that the symbiotic fungi of wheat, barley, and English ryegrass contain the nitrogenous compounds, especially prolamin, now known to be present in the kernels of these grasses.

EXPERIMENTAL WORK

MATERIALS

Inasmuch as English ryegrass was the plant in whose seeds symbiotic fungi were investigated especially by Freeman (*3*, *4*, *5*) and McLennan (*19*) and extensively studied by Peklo (*27*, *28*, *29*, *30*), a more thorough knowledge of its constituents seemed desirable for the purpose of comparing them with the constituents of the symbiotic fungi isolated from it. The English ryegrass seed employed in this investigation was kindly furnished by the Office of Forage Crops, Bureau of Plant Industry, and was of the 1922 crop. The writers are indebted to members of the Seed Laboratory of this bureau for determining the germination of the seed and the foreign seed contained in it. The English ryegrass used showed 15 per cent germination on May 5, 1927. It contained 99.09 per cent of pure seed, 0.58 per cent of inert matter, and 0.33 per cent of foreign seed. The kinds and numbers of foreign seed in a pound were as follows: Weed seeds, 902 (rat-tail fescue grass, 574; soft chess, 164; velvet grass, 82; and suckling clover, 82); and other seed, 246 (alsike clover, 164; crested dog's-tail, 82).

Because of the comparatively high purity of the seed, with which no trials requiring germination were contemplated, it was suitable for the writers' experiments. No special effort was made to separate the chaff from the whole of the seed, because the small size of the seed made this difficult. It was dried at 55° C. for two or three days, then repeatedly ground in an electric coffee mill until all the material passed through a 60-mesh sieve. The flour thus obtained was put into jars ready for use.

PREPARATION OF SYMBIOTIC FUNGI

The isolation of the symbiotic fungi from seeds of English ryegrass, wheat, and barley, as well as their inoculation into artificial culture media and their incubation, has already been described. After the expiration of a certain period the cultures from 1-liter Erlenmeyer flasks were partly filtered with suction on Büchner funnels containing two soft filters, and partly centrifuged whenever necessary. The solid fungus material was then repeatedly washed with water, next with water saturated with ether to wash out traces of the nutrient medium, next treated with ether to kill the fungus, and finally the whole was sucked as dry as possible. The fungus material, together with the filter, was then dried in the air, in a vacuum over sulphuric acid, or in an oven at about 45° C. In any case a part of the fungus tissue ordinarily sticks so tightly to the filter paper that it is practically impossible to separate it completely. The following procedure was applied successfully: Whenever the fungus could not be separated from the filter, both were ground in a mortar and passed through a 60-mesh or finer sieve, using several iron rings (washers) on the sieve. The grinding and the sifting were repeated several times if necessary. In this way a fungus powder containing some paper fiber was secured, while on the sieve remained the bulk of the paper fiber which was removed. To get rid of the rest of the paper fiber, the fungus powder containing some paper fiber was passed again through the sieve without the iron rings. This gave a practically pure fungus powder, while the rest of the paper fiber remained on the sieve.

METHODS

The ashing was effected in an electric muffle oven at dark-red heat until a constant weight was obtained. The total nitrogen was determined according to the Gunning modification of the Kjeldahl method. The protein nitrogen was estimated according to Stutzer's method (*33*) as outlined in former publications (*8*, *13*, *15*). Details concerning the estimation of the basic nitrogen in the phosphotungstic acid precipitate can also be found in previous publications (*10*, *14*, *16*). The determination of the nitrogen of acid amides, amino acids, and polypeptides was effected according to methods described in former papers (*9*, *11*, *12*).

ANALYSIS

A glance at Table 1, which contains some general information about English ryegrass, shows considerable differences in the moisture content of the English ryegrass seed. Thus, Nos. 1 to 3 have a lower average moisture content (4.22 per cent) than Nos. 4 and 5 (7.53 per cent) and Nos. 6 and 7 (7.96 per cent). The reason is that Nos. 4 to 7 represent strictly air-dry ryegrass, whereas Nos. 1 to 3,

to which the bulk of the seed belonged, were dried for two to three days at 55° C. in order to stop the activities of the enzymes and to have a material suitable for grinding and sifting and hence for the analytical and other work intended.

TABLE 1.—*Percentages of moisture, dry substance, ash, and ether extract in English ryegrass seed*

No.[a]	Date of analysis (1927)	Moisture in air-dry material	Dry substance in air-dry material	Ash in—		Ether extract in—		Remarks
				Air-dry material	Oven-dried material	Air-dry material	Oven-dried material	
1	Apr. 30	4.15	95.85	4.32	4.51	2.00	2.08	Containing some chaff.
2	do	4.28	95.72	4.30	4.49	2.01	2.09	Do.
3	do	4.23	95.77	4.29	4.47	2.01	2.09	Do.
Average		4.22	95.78	4.30	4.49	2.01	2.09	
4	June 30	7.65	92.35					Chaff free.
5	do	7.40	92.60					Do.
Average		7.53	92.47					
6	Sept. 20	7.97	92.03					Do.
7	do	7.95	92.05					Do.
Average		7.96	92.04					

[a] Nos. 1, 2, and 3 were dried for 2 to 3 days at 55° C.

English ryegrass seed has a fairly high average percentage (4.49) of ash in the oven-dried substance, as compared with seeds of other grasses—1.79 to 2.11 per cent in rye (*16*) and 3.15 to 3.72 per cent in oats (*9*). Upon analysis the ash of *Lolium perenne* was found to contain sodium (very little), potassium, calcium, magnesium, iron (ferric), manganese, phosphates, and sulphates (trace).

English ryegrass contains 2.09 per cent of ether extract calculated on the basis of the oven-dried material. This percentage compares favorably with 1.85 per cent of ether extract in wheat (*1, Bd. 1, p. 711*).

From Table 2 differences in the moisture content of the symbiotic fungi can be noticed. Thus, the English ryegrass fungus has a lower moisture content and hence a higher dry substance than either the wheat or the barley fungus. This is chiefly due to the fact that all the ryegrass fungus was prepared in Prague, Czechoslovakia, where it was kept in desiccators for some time prior to its shipment to Washington. Here, too, the fungus was allowed to remain in desiccators several weeks before it could be used for the experiments. Despite the fact that the wheat fungus grew in October (Nos. 6 and 7) faster than it did in the period from July 19 to September 16, 1927 (Nos. 4 and 5)—probably because the younger subcultures used for inoculation were also much younger than the first cultures—there is no considerable difference in their moisture content. Nor is there any noteworthy difference between the moisture percentage of the barley fungus cultivated in October (Nos. 10 and 11) and that cultivated from July 9 to September 7, 1927 (Nos 8 and 9), in spite of the fact that the former grew faster and was very much younger. In the symbiotic fungi the ash content is comparatively small; it is

highest in the English ryegrass fungus, lowest in the wheat fungus, and that of the barley fungus is between the two. For lack of material no fat estimations in the several symbiotic fungi in question could be made, though such determinations would be of certain interest, considering that fungi, yeasts, and bacteria are known to contain fats (*1, Bd. 3, p. 795*).

TABLE 2.—*Percentages of moisture, dry substance, and ash in the various symbiotic fungi*

No.	Date of analysis (1927)	Material used	Moisture in air-dry material	Dry substance in air-dry material	Ash in—		Remarks
					Air-dry material	Oven-dried material	
1	Sept. 26	English ryegrass fungus.	4.92	95.08	1.34	1.41	Cultures from 4 to 6 weeks old.
2	do	do	4.90	95.10	1.48	1.55	Do.
3	do	do	4.68	95.32			Do.
Average			4.83	95.17	1.41	1.48	Do.
4	Oct. 21	Wheat fungus	9.20	90.80			Grown from July 19 to Sept. 16, 1927.
5	do	do	9.70	90.30			Do.
Average			9.45	90.55			Do.
6	Nov. 17	Wheat fungus	7.50	92.50	1.00	1.08	Grown from Oct. 5 to 28, 1927.
7	do	do	7.40	92.60	1.20	1.30	Do.
Average			7.45	92.55	1.10	1.19	Do.
8	Oct. 22	Barley fungus	6.90	93.10			Grown from July 9 to Sept. 7, 1927.
9	do	do	7.10	92.90			Do.
Average			7.00	93.00			Do.
10	Nov. 19	Barley fungus	7.10	92.90	1.00	1.08	Grown from Oct. 20 to Nov. 1, 1927.
11	do	do	7.10	92.90	1.30	1.39	Do.
Average			7.10	92.90	1.15	1.24	Do.

Table 3 shows that English ryegrass seed has an average total nitrogen content ranging from 1.79 to 1.83 per cent calculated on the basis of the oven-dried seed, an average protein nitrogen ranging from 87.93 to 91.49 per cent calculated on the total nitrogen, and an average nonprotein nitrogen ranging from 8.51 to 12.07 per cent calculated on the total nitrogen. Although the nonprotein nitrogen was calculated by taking the difference from 100, it was ordinarily checked by direct nitrogen estimations in the filtrates from the protein-copper precipitates as obtained by Stutzer's method (*33*) and found to be reasonably accurate. In order to have a clearer conception of what the figures in Table 3 mean, it seems best to compare them with those of other well-known grasses, especially of wheat (*14*), rye (*16*), and oats (*9*). The figures under consideration are presented in Table 4.

From Table 4 it will be seen that the total nitrogen of English ryegrass is about equal to that of Fultz wheat and Von Rümker rye, higher than that of registered Rosen rye and Winter Turf oats, but lower than that of Victory oats. The protein nitrogen of English

ryegrass based on the total nitrogen is about equal to that of all the grasses mentioned with the exception of Von Rümker rye, which has a lower protein content. The nonprotein nitrogen stands in reverse ratio, as would be expected.

In connection with Tables 3 and 4 it may not be out of place to mention that Schulze (*32, p. 110–111*) has reported the nitrogen percentages of two varieties of Italian ryegrass to be as follows: First variety—total nitrogen, 2.35; protein nitrogen, 1.81; nonprotein nitrogen, 0.54; second variety—total nitrogen, 3.64; protein nitrogen, 3.04; nonprotein nitrogen, 0.60. These figures are very much higher than those recorded in Tables 3 and 4.

TABLE 3.—*Percentages of total, of protein, and of nonprotein nitrogen in English ryegrass seed*

No.	Date of analysis (1927)	Total nitrogen on the basis of—		Protein nitrogen on the basis of—		Nonprotein nitrogen, by difference from 100, on the basis of—		Remarks
		Air-dry substance	Oven-dried substance	Oven-dried substance	Total nitrogen	Oven-dried substance	Total nitrogen	
1	May 7	1.73	1.80	1.61	90.00	0.179	10.00	Containing some chaff.
2	do	1.67	1.76	1.49	83.18	.301	16.82	Do.
3	do	1.72	1.80	1.62	90.62	.168	9.38	Do.
Average		1.71	1.79	1.57	87.93	.216	12.07	
4	June 30	1.66	1.80					Chaff free.
5	do	1.71	1.85					Do.
Average		1.69	1.83					
6	Sept. 30	1.71	1.86					Do.
7	do	1.65	1.80	1.67	91.49	.156	8.51	Do.
Average		1.68	1.83	1.67	91.49	.156	8.51	

TABLE 4.—*A comparison of the nitrogen percentages of English ryegrass with those of other grasses*

No.	Name		Total nitrogen on the basis of—		Protein nitrogen on the basis of—		Nonprotein nitrogen, by difference from 100, on the basis of—	
	Grass	Variety	Air-dry substance	Oven-dried substance	Oven-dried substance	Total nitrogen	Oven-dried substance	Total nitrogen
1	English ryegrass	Of Irish origin	1.68 to 1.71	1.79 to 1.83	1.57 to 1.67	87.93 to 91.49	0.156 to 1.216	8.51 to 12.07
2	Wheat	Fultz		1.80	1.64	91.76		8.24
3	Rye	Von Rümker		1.84	1.58	85.73	.26	14.27
4	do	Registered Rosen		1.65	1.51	91.52	.14	8.48
5	Oats	Victory		2.02	1.82	90.21	.20	9.79
6	do	Winter Turf		1.50	1.36	91.03	.14	8.97

Reference to Table 5 reveals the fact that of the symbiotic fungi the English ryegrass fungus has a remarkably high average nitrogen content, namely, 4.57 per cent, calculated on the oven-dried fungus. The barley fungus has the second-highest average nitrogen content, 3.51 (Nos. 8 and 9) and 3.35 per cent (Nos. 10 and 11), while the wheat fungus has the lowest average nitrogen content, 1.83 (Nos. 4 and 5) and 2.44 per cent (Nos. 6 and 7). It is worthy of note that

the wheat fungus that was grown for but 24 days (Nos. 6 and 7) has a higher average nitrogen content (2.44 per cent) than that grown for about two months (1.83 per cent, in Nos. 4 and 5). The reverse, however, holds true for the barley fungus, since the cultures grown for but 12 days (Nos. 10 and 11) have a lower average nitrogen content (3.35 per cent) than the cultures grown for about two months (3.51 per cent, in Nos. 8 and 9), calculated on the oven-dried substance. In this connection it may be pointed out that under certain conditions the barley fungus evidently does not fully utilize the nitrogen offered in the nutrient medium or loses some of its nitrogen in the form of crystals.

Concerning the protein nitrogen, it will be seen that the barley fungus has the highest percentage, calculated on the basis of the total nitrogen, while the protein nitrogen in the ryegrass fungus and wheat fungus, which are equal in this respect, is about 11 per cent smaller than in the barley fungus. The nonprotein nitrogen of the different fungi stands in reverse ratio to the protein nitrogen, as usual. The great differences in the total and protein nitrogen of the symbiotic fungi rather emphasize the necessity of finding out optimum conditions for their growth, not only with regard to the duration of cultivation but also with regard to temperature, nutrient media, and other factors.

TABLE 5.—*Percentages of total, of protein, and of nonprotein nitrogen in the various symbiotic fungi*

No.	Date of analysis (1927)	Material used	Total nitrogen on basis of—		Protein nitrogen on basis of—		Nonprotein nitrogen by difference from 100, on basis of—		Remarks
			Air-dry fungus	Oven-dried fungus	Oven-dried fungus	Total nitrogen	Oven-dried fungus	Total nitrogen	
1	Sept. 23	English ryegrass fungus.	4.34	4.56	3.62	79.24	0.95	20.76	Cultures from 4 to 6 weeks old.
2	do	do	4.29	4.50	3.66	80.16	.91	19.84	Do.
3	do	do	4.44	4.66					Do.
Average			4.36	4.57	3.64	79.70	.93	20.30	
4	Oct. 21	Wheat fungus	1.64	1.82	1.44	78.96	.51	21.04	Grown from July 19 to Sept. 16, 1927.
5	do	do	1.67	1.84	1.45	79.46	.50	20.54	Do.
Average			1.66	1.83	1.45	79.21	.51	20.79	
6	Nov. 19	do	2.22	2.39					Grown from Oct. 5 to Oct. 28, 1927.
7	do	do	2.29	2.48					Do.
Average			2.26	2.44					
8	Oct. 22	Barley fungus	3.24	3.48	3.19	90.81	.32	9.19	Grown from July 9 to Sept. 7, 1927.
9	do	do	3.28	3.53	3.22	91.67	.29	8.33	Do.
Average			3.26	3.51	3.21	91.24	.31	8.76	
10	Nov. 19	do	3.09	3.32					Grown from Oct. 20 to Nov. 1, 1927
11	do	do	3.14	3.38					Do.
Average			3.12	3.35					

NATURE OF CRYSTALS FORMED IN CULTURES OF BARLEY FUNGUS

The following observation appears to substantiate the statement that nitrogen is lost in the form of crystalline compounds. After the barley fungus had grown in A. Meyer's solution plus 1 per cent dextrose and 1 per cent peptone for about two months and the contents of the culture flasks were collected on filters in Büchner funnels, it was observed that white rhombic crystals remained on the walls of the flasks. The crystals were examined for asparagine and calcium oxalate, with negative results. They were practically insoluble in water and alcohol, but soluble in dilute hydrochloric acid, from which they were reprecipitated on the addition of ammonia. On being treated with ammonium molybdate solution they gave the yellow precipitate characteristic of phosphoric acid. When heated with sodium hydroxide solution they evolved ammonia. These reactions showed that the crystals under examination were magnesium ammonium phosphate. In younger cultures of the barley fungus the crystals were also found, though in smaller quantities; but no crystals could be observed in 12-day-old cultures with 2 per cent of dextrose. The occurrence of the crystals mentioned, containing ammonia, involves a certain loss of protein in the pure cultures of the barley fungus when cultivated too long. A similar loss may occur in the analogous cultures of the wheat fungus.

NATURE OF THE PROTEINS

In order to get some general idea of the nature of the proteins present in the English ryegrass, the writers made a series of extractions whose arrangements and results can best be seen from Table 6.

Table 6 needs some explanation. In the case of extractions Nos. 1, 2, 3, 4, 5, and 6 the quantity of flour used was uniformly 100 gm., and it was extracted with 500 c. c. of the liquid specified at room temperature. In each case 2 c. c. of toluol was added to prevent bacterial action. The flasks were shaken frequently. After the expiration of 36 hours extracts Nos. 1, 3, 5, and 6 were filtered through Büchner funnels provided with two soft filters. Extracts Nos. 2 and 4 had to be filtered first through cloth, which was afterwards pressed out in a screw press. The turbid liquids thus obtained were then centrifuged. Of the final filtrates 100 c. c. portions were oxidized according to the Kjeldahl method. Extraction No. 1a was obtained differently; six flasks each containing 25 gm. of flour were treated with 500 c. c. of boiling-hot, ammonia-free water and kept on the water bath for 30 minutes, after which their contents were partly filtered on Büchner funnels and partly centrifuged. The solid residues were extracted once more in like manner. The filtrates were then concentrated in vacuo, centrifuged again, and finally made up to 500 c. c., of which two portions of 200 c. c. were Kjeldahlized. The results obtained will be found in the last two columns of Table 6.

Examination of Table 6 shows that distilled water at room temperature extracted more nitrogen (16.16 per cent) than boiling water (7.09 per cent). Since water ordinarily extracts albumins and, because of the presence of salts and globulins in plant materials, the difference may be explained by the assumption that the heat applied

to extract No. 1a very likely led to the coagulation of most of the albumin and perhaps of some globulin. Proteose, which is usually present in insignificant quantities in plant extracts, may be left out of consideration.

TABLE 6.—*Percentage of nitrogen extracted from English ryegrass by different solvents at various temperatures*

Extraction No.	Flour used (grams)	Solvents used for extraction			Temperature employed	Duration of extraction (hours)	Percentage of nitrogen extracted, calculated on the basis of—	
		Name	Per cent	Quantity (c. c.)			Oven-dried flour	Total nitrogen
1	100	Distilled water		500	Room	36	0.289	16.16
1a	150	do		3,000	100° C.	1	.127	7.09
2	100	Potassium hydroxide	0.2	500	Room	36	.848	47.38
3	100	Hydrochloric acid	.2	500	do	36	.256	14.31
4	100	Sodium chloride	10	500	do	36	.350	19.54
5	100	Alcohol	70	500	do	36	.307	17.13
6	100	do	85	500	do	36	.098	5.45

The 10 per cent sodium chloride solution, which extracts primarily globulins but also albumins and proteoses if they are present, extracted 19.54 per cent of nitrogen. If extracts Nos. 1 and 1a represent chiefly albumin nitrogen, which is reasonable, it follows from extract No. 4 (19.54 per cent nitrogen) that there is left a safe margin for the presence of globulin nitrogen in the ryegrass.

From extractions Nos. 5 and 6 it follows plainly that 70 per cent alcohol extracts considerably more nitrogen (prolamin) than 85 per cent alcohol does.

A solution of 0.2 per cent potassium hydroxide extracts primarily glutelins, but also the other proteins if present. Assuming that Nos. 5 and 4 represent the prolamin, globulin, and albumin nitrogen, it follows from the large proportion of nitrogen extracted with 0.2 per cent potassium hydroxide solution (47.38 per cent in No. 2) that there is still left a considerable margin for the presence of glutelin in the ryegrass. This conclusion was actually corroborated in the following way. A portion of ryegrass flour was first completely extracted with 70 per cent alcohol and then with 10 per cent sodium chloride solution. The remaining residue was then treated with 0.2 per cent potassium hydroxide solution. The extract thus obtained, which must have been glutelin, gave the usual protein reactions.

Extraction No. 3 shows that 0.2 per cent hydrochloric acid solution extracts very much less nitrogen than 0.2 per cent potassium hydroxide.

TESTING EXTRACTS OF ENGLISH RYEGRASS FOR PROTEINS

While the results in Table 6 appear to indicate the presence of albumins, globulins, prolamins, and glutelins in the English ryegrass, it should be borne in mind that the solvents used may also extract nonprotein nitrogenous compounds. Hence, it seems necessary to corroborate by other methods the conclusions drawn. First, it

appears imperative to demonstrate that the extracts obtained by the various solvents actually represent protein nitrogens in each and every case. This was done in the following way: Extracts Nos. 1 to 4 (Table 6) were treated with solid ammonium sulphate until a maximum precipitate was obtained; next they were centrifuged, and the supernatant liquids were removed, leaving the precipitates on the bottom of the centrifuge tubes. Extracts Nos. 5 and 6 were diluted with several volumes of water until a maximum precipitate resulted. All the precipitates thus obtained were tested for proteins, with the results given in Table 7. It may be added that some of the extracts gave directly, without previous concentration, positive protein reactions.

OCCURRENCE OF ANTHOCYANIN IN ENGLISH RYEGRASS

When the ryegrass-seed flour was extracted with 70 per cent alcohol it was noticed that the extract gradually assumed a brown-reddish color that intensified on standing. It was but natural to think of the anthocyanin pigment. In order to obtain definite information as to the nature of the pigment the following reactions were made: (1) When acids were added to the pigment extract it turned red; (2) on rendering the extract alkaline with sodium hydroxide it assumed a bluish color, which, on the addition of more alkali, turned green; (3) when the extract was treated with zinc and sulphuric acid it became finally colorless through the action of the nascent hydrogen, but on exposure to air the color returned again; and (4) the pigment appeared to be unstable, as shown by the fact that strong acids, especially on heating, readily destroyed the pigment. These reactions show that the pigment is anthocyanin.

TABLE 7.—*Examination of the various extracts of English ryegrass for proteins*

No.	Solvents used for extraction		Methods of concentrating the protein	Reaction to—				
	Name	Per cent		Millon's reagent	Biuret test	Xantho-protein test	Molisch test	Pauly's diazo reagent
1	Distilled water		Precipitation with ammonium sulphate.	Positive (strong).		Positive.	Positive.	Positive.
2	Potassium hydroxide.	0.2	do	do		do	do	Positive (strong).
3	Hydrochloric acid	.2	do	do	Positive.	Positive (weak).	do	Do.
4	Sodium chloride	10	do	do	do	do	do	Do.
5	Alcohol	70	Dilution with water.	do	do	Positive (strong).	do	Positive.
6	do	85	do	do	Negative.	do	do	Positive (strong).

QUANTITATIVE ESTIMATION OF PROLAMIN NITROGEN IN ENGLISH RYEGRASS SEED

From Table 6 (extracts Nos. 5 and 6) it was evident that English ryegrass contains alcohol-soluble nitrogen, which was shown to be chiefly protein nitrogen (Table 7). The fact, however, that alcohol may extract not only prolamins but also nonprotein nitrogenous compounds made it imperative to apply a method by which the pro-

lamin nitrogen only could be estimated. A modification of Stutzer's method (*33*) was found to be accurate for this purpose. The procedure was as follows: (*a*) 20 gm. of ryegrass-seed flour was weighed in a 1-liter flask, to which 500 c. c. of 70 per cent alcohol was added and shaken frequently. After standing for 24 hours at room temperature the flask was again shaken well and the contents filtered through a dry filter until perfectly clear. Of this filtrate two portions of 50 c. c. each were oxidized according to the Kjeldahl method to determine the total alcohol-soluble nitrogen present. Two or three other portions of 100 c. c. were used for the determination of the prolamin nitrogen, while the rest of the filtrate was employed for qualitative protein tests and for polariscopic observations. Two more 20 gm. portions (*b*) and (*c*) of ryegrass-seed flour were treated exactly as in *a* except that they were allowed to stand for 48 hours (*b*) and 72 hours (*c*), respectively. A fourth portion (*d*) was extracted at 60° C. for 24 hours.

TABLE 8.—*Determination of the percentages of prolamin nitrogen in the extract from 20 gm. of air-dry English ryegrass seed using 500 c. c. of 70 per cent alcohol*

Experiment	Hours extracted	Temperature at which extracted	Total alcohol-soluble nitrogen found, calculated on the basis of—		Prolamin nitrogen found, calculated on the basis of—	
			Oven-dried seed	Total nitrogen	Oven-dried seed	Total nitrogen
A	24	Room	{ 0.46 .45	{ 25.33 24.73	0.35 .33	18.99 18.25
Average			.46	25.03	.34	18.62
B	48	Room	{ .44 .43	{ 24.17 23.77	.37 .33	20.37 17.81
Average			.44	23.97	.35	19.09
C	72	Room	{ .47 .55	{ 25.85 29.85	.31 .30	17.06 16.51
Average			.51	27.85	.31	16.78
D	24	60° C.	{ .64 .67	{ 35.02 36.85	.52 .52	28.33 28.37
Average			.66	35.94	.52	28.35

The prolamin nitrogen proper was estimated as follows: Two or three 100 cm. portions of the alcoholic extract were separately evaporated on the water bath until all the alcohol was removed. Each residue, in a separate beaker, was then made up with distilled water to 100 c. c. Then it was heated to the boiling point and allowed to stay on the steam bath for 10 minutes. Next each beaker received 2 c. c. of saturated alum solution and some excess of Stutzer's reagent (gylcerol copper hydroxide emulsion (*33*)). The latter reagent was added gradually with constant stirring. Each beaker was then allowed to cool in a refrigerator. Next its contents were separately decanted on a Büchner funnel provided with two soft snugly fitting filters, and the decantation was hastened by means of suction. To the residue 100 c. c. of ice-cold water was added, and the whole was

stirred well and allowed to stay in the refrigerator until the precipitate had settled. The supernatant liquid was then again decanted on the Büchner, and this operation was repeated two or three times. Finally the precipitate was quantitatively transferred to the filter and washed with ice-cold water. The last washing was tested with barium chloride solution for the absence of sulphate. All the filtrations and washings usually took less than one hour. The precipitate together with the filters, which should preferably be nitrogen free, was then oxidized according to the Kjeldahl method, whereby the addition of 45 c. c. of concentrated sulphuric acid plus 10 gm. of potassium sulphate was found satisfactory for the oxidation. The nitrogen found here minus the nitrogen estimated in a blank gives the prolamin nitrogen. When the operations as outlined here are made carefully, closely agreeing duplicates are usually obtained. The results are summarized in Table 8.

Examination of Table 8 reveals the fact that the proportions of the total alcohol-soluble nitrogen and the prolamin nitrogen extracted in 24 and 48 hours, respectively, are almost identical, while in 72 hours more alcohol-soluble nitrogen and less prolamin nitrogen were extracted. Strikingly greater is the proportion of both forms of nitrogen extracted at 60° C. Thus, the percentages of total alcohol-soluble nitrogen extracted in 24, 48, and 72 hours at room temperature are, on an average, respectively, 25.03, 23.97, and 27.85, as compared with 35.94 per cent at 60° C., calculated on the basis of the total nitrogen. Likewise, the average percentages of prolamin nitrogen in 24, 48, and 72 hours at room temperature are, respectively, 18.62, 19.09, and 16.78, as compared with 28.35 per cent obtained at 60° for 24 hours, calculated on the basis of the total nitrogen. Of special importance is the fact that in each and every case 70 per cent alcohol extracted considerable proportions of prolamin from the English ryegrass seed.

Mention may be made here of the fact that an alcoholic extract of English ryegrass seed, which was concentrated in a vacuum, yielded on some purification a substance that gave the usual protein reactions and behaved otherwise as a prolamin. However, the complete isolation, purification, and identification will require considerable time. Owing to the fact that Peklo had to leave Washington for Prague to resume his duties there, it was necessary to postpone this work and otherwise to limit the scope of this paper. It is hoped, however, that this work, as well as other phases of this investigation, will be taken up later.

QUANTITATIVE ESTIMATION OF PROLAMIN NITROGEN IN THE SYMBIOTIC FUNGI

Because the writers had only very small quantities of the wheat and barley fungi, no exhaustive experiments to determine optimum conditions for extracting maximum proportions of nitrogen of the symbiotic fungi by means of alcohol could be made. However, since more of the English ryegrass fungus was available, it was decided to use this fungus in a few preliminary experiments to determine the influence of temperature, time, and other factors on the extractability of the nitrogen and to apply the results obtained to the other two fungi. The arrangement of the experiments can best be seen from Table 9.

TABLE 9.—*Percentages of nitrogen extracted from English ryegrass fungus by 70 per cent alcohol under varying conditions*

No.	Fungus powder used (grams)		Extraction of fungus powder with 70 per cent alcohol			Alcoholic extract used for Kjeldahl (c. c.)	Nitrogen extracted, calculated on the basis of—	
	Air-dry	Oven-dried	Alcohol added (c. c.)	Duration of extraction (hours)	Temperature employed		Oven-dried fungus	Total nitrogen
1	14	13.3238	700	24	Room	100	0.67	14.92
						100	.69	15.21
Average							.68	15.06
2	14	13.3238	700	24	60° C	100	.88	19.38
						100	.90	19.74
Average							.89	19.56
3	1.4	1.3324	140	2	80° C	120	.95	20.78
4	1.4	1.3324	140	3½	80° C	120	1.01	22.09
5	1.4	1.3324	140	5	80° C	125	1.01	22.06

From Table 9 it is evident that the quantity of nitrogen extracted with 70 per cent alcohol increased with rising temperature. Thus, at room temperature, during 24 hours, 15.06 per cent of the total nitrogen was extracted, as compared with 19.56 per cent at 60° and with 20.78 per cent at 80° C. during but two hours. The influence of the time factor is evident from experiments 3, 4, and 5; in 2 hours at 80°, 20.78 per cent of nitrogen was extracted, as compared with 22.09 per cent in 3½ hours. However, extraction at 80° for 5 hours did not increase the quantity of nitrogen extracted. Consequently, it was decided to apply the temperature of 80° for 3½ hours to the extraction of the various fungi. Briefly stated, quantities of the symbiotic fungi ranging from 5 to 10 gm. were extracted with definite amounts of 70 per cent alcohol under reflux, with frequent shaking. At the expiration of 3½ hours the whole was filtered through a dry filter, using a hot-water funnel. The clear filtrate was divided into 50 or 100 c. c. portions, of which two were used for Kjeldahl determinations to find the proportion of the total alcohol-soluble nitrogen, two or more other portions were used for estimations according to Stutzer's modified method to ascertain the proportion of prolamin nitrogen, and the rest of the filtrate was used for qualitative protein tests and for polariscopic observations. The details of the determination of the prolamin nitrogen present in the fungi will be found in the estimation of the prolamin nitrogen as applied to the English ryegrass seed. The results are presented in Table 10.

A glance at Table 10 shows that 70 per cent alcohol extracted considerable percentages of nitrogen from the symbiotic fungi, it being highest in the ryegrass fungus (21.91) and lowest in the barley fungus (11.68), while the wheat fungus is between the two (21.1), calculated on the basis of the total nitrogen. So far as the prolamin nitrogen is concerned, it will be seen that it is again highest in the ryegrass fungus (9.51 per cent), followed by the fungi of wheat (6.47 per cent), and barley (5.35 per cent) in the order named, when calculated on the total nitrogen.

TABLE 10.—*Quantitative determination of prolamin nitrogen in the symbiotic fungi digested with 70 per cent alcohol for 3½ hours at 80° C.*

Experiment No.	Fungous powder		Alcohol added (c. c.)	Per cent of total alcohol-soluble nitrogen found, calculated on the basis of—		Per cent of prolamin nitrogen found, calculated on the basis of—	
	Kind	Air-dry fungus (gm.)		Oven-dried fungus	Total nitrogen	Oven-dried fungus	Total nitrogen
1	English ryegrass fungus	5	500	0.97	21.13	0.41	8.92
2						.43	9.34
3						.46	9.99
4	do	5	500	1.04	22.68	.38	8.37
5						.42	9.28
6						.47	10.31
7						.47	10.37
Average				1.01	21.91	.43	9.51
8	Wheat fungus	10	800	.35	19.07	.11	6.09
9				.42	23.13	.12	6.56
10						.12	6.77
Average				.39	21.10	.12	6.47
11	Barley fungus	7	700	.38	10.82	.20	5.83
12				.44	12.53	.21	5.92
13						.15	4.29
Average				.41	11.68	.19	5.35

A portion of the alcoholic extract of each of the symbiotic fungi was evaporated on the water bath and the residues tested for protein, with the results given in Table 11.

The remainder of the alcoholic extracts of the fungi and of English ryegrass seed was used, as stated, for polariscopic observations. However, the rotations observed were too small to be considered sufficiently reliable and are omitted here. Polariscopic observations will have to be repeated with more concentrated extracts to secure dependable results.

TABLE 11.—*Examination of the alcoholic extracts of symbiotic fungi for proteins*

Source of fungus	Reaction to—			
	Millon's reagent	Xanthoprotein test	Pauly's diazo reagent	Biuret test
English ryegrass	Positive	Positive (strong)	Positive (strong)	Positive.
Wheat	do	Positive	Positive	
Barley	do	Positive (strong)	do	

A CARBOHYDRATE OCCURRING IN THE SYMBIOTIC FUNGI

When the symbiotic fungi of ryegrass, wheat, and barley were extracted with 70 per cent alcohol at 80° C. for the purpose of obtaining the alcohol-soluble nitrogen, and the extract was allowed to stand for some time, preferably in the refrigerator, it was noticed that a yellowish white, more or less flocculent precipitate formed. When the extract was placed on the steam bath the precipitate redissolved

in the alcohol but reappeared on cooling. The precipitate was separated from the alcoholic liquid by centrifugation and washed repeatedly with water.

In order to obtain some information concerning its nature, several reactions were applied to the precipitate, with the following results: (1) Protein reactions were negative; (2) the Molisch reaction was strongly positive; (3) Trommer's reaction (with Fehling's solution) was negative; and (4) on hydrolysis, Trommer's reaction was positive. The substance was hydrolyzed as follows: The precipitate was boiled for two hours with 4.5 per cent hydrochloric acid, using a reflux condenser. When the hydrolysate was neutralized with sodium hydroxide and boiled with Fehling's solution a strong reduction to cuprous oxide took place. It was ascertained that this reduction was caused by the substance in solution, since the filtrate alone gave the same reduction. The substance under examination is not starch, judging from its solubility in hot alcohol and its gelatinous appearance. It is apparently a carbohydrate, perhaps in combination with some other compound. Not until more material has been secured will it be possible to ascertain the exact nature of the substance.

In this connection it is interesting to recall that cereal seeds have also been found to contain similar carbohydrates. Thus, Ritthausen (*31, p. 96*) has reported the occurrence in the rye seed of a gum which is soluble in dilute alcohol and water and has the formula $C_6H_{10}O_5$. According to Frankfurt (*2, p. 457*) the wheat embryo contains considerable quantities of sucrose and raffinose as well as some glucose, while, according to Hoffman and Gortner (*7, p. 226*), wheat flour contains a gum.

NATURE OF THE NONPROTEIN NITROGEN OCCURRING IN ENGLISH RYEGRASS

Table 3 shows that English ryegrass contains on the average from 8.51 to 12.07 per cent of nonprotein nitrogen, calculated on the basis of the total nitrogen. In order to gain an insight into the character of the organic nitrogenous compounds occurring in the ryegrass, the writers proceeded as follows: The filtrate from the copper-protein precipitate obtained by the Stutzer method (*33*) was treated with hydrogen sulphide, the copper sulphide filtered off, and the filtrate evaporated on the water bath. The concentrated solution was freed by filtration from the copper sulphide, which had separated during evaporation, and finally made up to 100 c. c. Of this solution 10 c. c. was oxidized according to the Kjeldahl method to ascertain the total nitrogen present. The remaining 90 c. c. was made up with water to 100 c. c., to which was added 5 gm. of concentrated sulphuric acid and some excess of a phosphotungstic acid solution containing 20 gm. of phosphotungstic acid and 5 gm. of sulphuric acid per 100 c. c. After 24 hours the precipitate was filtered off and washed with a solution containing 2.5 gm. of phosphotungstic acid and 5 gm. of sulphuric acid per 100 c. c. The washed precipitate was then oxidized according to the Kjeldahl method, a procedure which yielded the "basic" nitrogen. It should be borne in mind, however, that the phosphotungstate precipitate contains not only diamino acids and other basic compounds but also proteoses and peptones, if present.

To the filtrate and washings from the phosphotungstate precipitate calcium hydroxide was added to slight acidity; then barium hydroxide was added to distinct alkalinity. The whole was then saturated with carbon dioxide, boiled for several minutes, and filtered on a Büchner funnel provided with a hardened filter. The cake that remained on the Büchner funnel was extracted once more with hot water from which the ammonia had been removed by long boiling. The combined extracts and washings were then concentrated by evaporation and finally made up to 100 c. c., and 10 c. c. of this solution was oxidized by the Kjeldahl method to determine the total nitrogen present. To the remaining 90 c. c. enough concentrated hydrochloric acid was added to make a 20 per cent solution and the mixture was boiled in a glycerin bath for half an hour at about 130° C., using a reflux condenser. The hydrolysate was then almost neutralized with sodium hydroxide and distilled with magnesium oxide previously reduced to cream with water. The titration of the distillate gave the ammonia nitrogen corresponding to the acid amides present.

The residue in the Kjeldahl flask, after the ammonia had been driven off, was first decanted through a Büchner filter and then treated a number of times with boiling-hot ammonia-free water, the contents being decanted each time through the Büchner. Finally the residue was transferred to the filter and washed repeatedly with hot water. As the filtrates and washings were concentrated on the water bath a large quantity of crystals, which were identified as sodium chloride, appeared. The mother liquor was expressed from the crystals by crushing them finely, treating them with strong alcohol, filtering with suction, and washing with alcohol. On concentrating the alcoholic filtrates and washings on the water bath, two more crops of sodium chloride crystals appeared. They were also treated in the manner described. Finally, the solution freed from the crystals was made up to 100 c. c. The total nitrogen present was determined in 10 c. c. of this solution by the Kjeldahl method. The remaining 90 c. c. was made up to 100 c. c. with water, and divided into two portions of 50 c. c. each, one portion being used for the estimation of the amino acid nitrogen by the formol titration method, the other for the determination of peptide nitrogen. The latter determination was effected as follows: To the 50 c. c. portion concentrated hydrochloric acid was added to a concentration of 20 per cent and the solution was boiled in a glycerin bath for 12 hours, using a reflux condenser. The hydrolysate was next evaporated to dryness on the water bath; the residue was taken up with hot water, and made to volume at 100 c. c., of which 20 c. c. was used for a total nitrogen determination by the Kjeldahl method and 50 c. c. for the estimation of the amino nitrogen by the formol titration method. The peptide nitrogen value is obtained by subtracting from this result the amino nitrogen, which was present as such in the solution. The results secured by the methods described, which were also applied to the symbiotic fungi, are presented in Table 12.

By reference to Table 12 it will be seen that the nonprotein nitrogen of the English ryegrass is made up of 3.68 and 35.74 per cent of basic nitrogen, and of 1.86 and 18.06 per cent of acid amide nitrogen calculated, respectively, on the basis of the total and nonprotein nitrogen, while only a trace of amino nitrogen and no peptide nitrogen were found. So far as the English ryegrass fungus is concerned,

it will be noticed that in it the basic nitrogen (39.83 per cent), acid amide nitrogen (5.49 per cent), and amino nitrogen (51.95 per cent) make up practically the whole of the nonprotein nitrogen. In the wheat fungus the basic and acid amide nitrogen make up but 3.73 and 11.92 per cent of the nonprotein nitrogen, respectively, while amino and peptide nitrogen were entirely absent. In the barley fungus, because of accidental loss, only one estimation was made, namely, that of the basic nitrogen, which was found to make up 10.03 per cent of the nonprotein nitrogen.

TABLE 12.—*Distribution of the nonprotein nitrogen in English ryegrass and symbiotic fungi*

Material used	Nitrogen of the phosphotungstate precipitate (basic nitrogen), calculated on the basis of—			Acid amide nitrogen, calculated on the basis of—			Amino acid nitrogen, calculated on the basis of—		Peptide nitrogen
	Nonprotein nitrogen	Total nitrogen	Oven-dried material	Nonprotein nitrogen	Total nitrogen	Oven-dried material	Nonprotein nitrogen	Total nitrogen	
	Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
English ryegrass seed	35.74	3.68	0.066	18.06	1.86	0.034	Trace.	Trace.	0
English ryegrass fungus	39.83	8.09	.370	5.49	1.12	.051	51.95	10.55	0
Wheat fungus	3.73	.775	.019	11.92	2.48	.061	0	0	0
Barley fungus	10.03	.879	.031						

SUMMARY

The cytological observations of the junior writer have led to the conclusion that symbiotic fungi of English ryegrass, wheat, and barley produce the aleurone layer and later penetrate also the peripheral cells of the endosperm, leaving there the proteins present in their tissues, the so-called gluten.

The symbiotic fungi have been isolated from English ryegrass and certain kinds of wheat and barley, and identified as smuts.

Biochemically, it has been shown in this paper for the first time that English ryegrass seed contains an alcohol-soluble protein (prolamin). The percentages of prolamin nitrogen (extracted at 60° C.) were found to be 0.52 and 28.35, calculated on a basis of the oven-dried seed and its total nitrogen, respectively. The symbiotic fungi of English ryegrass, wheat, and barley have been shown to contain the proteins known to occur in the seeds of English ryegrass, wheat, and barley.

Of special interest seems to be the fact shown for the first time in this paper that each of the above-mentioned symbiotic fungi contains prolamin, an essential component of gluten. The proportions of prolamin nitrogen found were as follows: In the English ryegrass fungus, 0.43 and 9.51 per cent; in the wheat fungus, 0.12 and 6.47 per cent; in the barley fungus, 0.19 and 5.35 per cent, calculated on the basis of the oven-dried fungus and of its total nitrogen, respectively.

The nonprotein nitrogen contained in English ryegrass and in the symbiotic fungi was shown to be made up, in part, of protein degradation products, such as acids amides and amino acids.

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RHIZOCTONIA ROT OF TURNIPS IN STORAGE[1]

By J. I. LAURITZEN

Pathologist, Office of Horticultural Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture

INTRODUCTION

A disease, caused by a species of Rhizoctonia, has occurred each storage season since 1923–24 in turnips (*Brassica rapa* Linn.) grown and stored at the Arlington Experiment Farm, Rosslyn, Va. The disease also has been observed occasionally on the New York and other markets in turnips and rutabagas (*B. campestris* Linn.). This report is believed to be the first of the occurrence of such a disease of turnips in storage.

In 1902 Rolfs (*8*)[2] gave a list of hosts in which *Rhizoctonia solani* Kühn occurs. He included turnips in this list, but did not state the part of the plant in which it occurred. In a letter to Peltier (*7*), Clinton reported the occurrence of a field root rot of turnip caused by Rhizoctonia in Connecticut. Dana (*3*) found mycelium and sclerotia of *R. solani* and basidia of *Corticium vagum* B. and C. on growing turnip roots in Washington. Anderson and his coworkers (*1*) reported the occurrence of stem rot of turnips caused by *C. vagum* in Massachusetts, Ohio, and Washington.

The economic importance of the disease is not known except locally, where the losses have varied during the last four seasons from less than 1 to 87 per cent, depending upon the season and the conditions of storage.

The purposes of this paper are briefly as follows: (1) To give a description of the disease; (2) to present some observations and experimental data on (*a*) the causal organism, its pathogenicity and life history, (*b*) the temperature relations of the pathogene on culture media, (*c*) the losses occurring under certain storage conditions, (*d*) varietal susceptibility of the host, and (*e*) the humidity and temperature relations of infection and the development of the disease; and (3) to make recommendations for its control.

DESCRIPTION OF THE DISEASE

On turnips Rhizoctonia causes lesions varying from circular to elliptical in the surface outline. The decay generally extends inwardly toward the center of the root in a radial fashion (fig. 1, C), and becomes more or less cone shaped with the apex of the cone toward the center. The necrotic tissue is somewhat watery and more or less spongy. The consistency resembles that of Botrytis decay of turnip and carrot, but is slightly less firm and not quite so watery. The decayed tissue internally is of a light-brown color, often interspersed with lighter areas, especially during the latter part of the storage season, when some of the normal tissue commences to collapse, much as sweet potatoes do in case of internal breakdown (*4*).

[1] Received for publication Sept. 19, 1928; issued February, 1929.

[2] Reference is made by number (italic) to "Literature cited," p. 108.

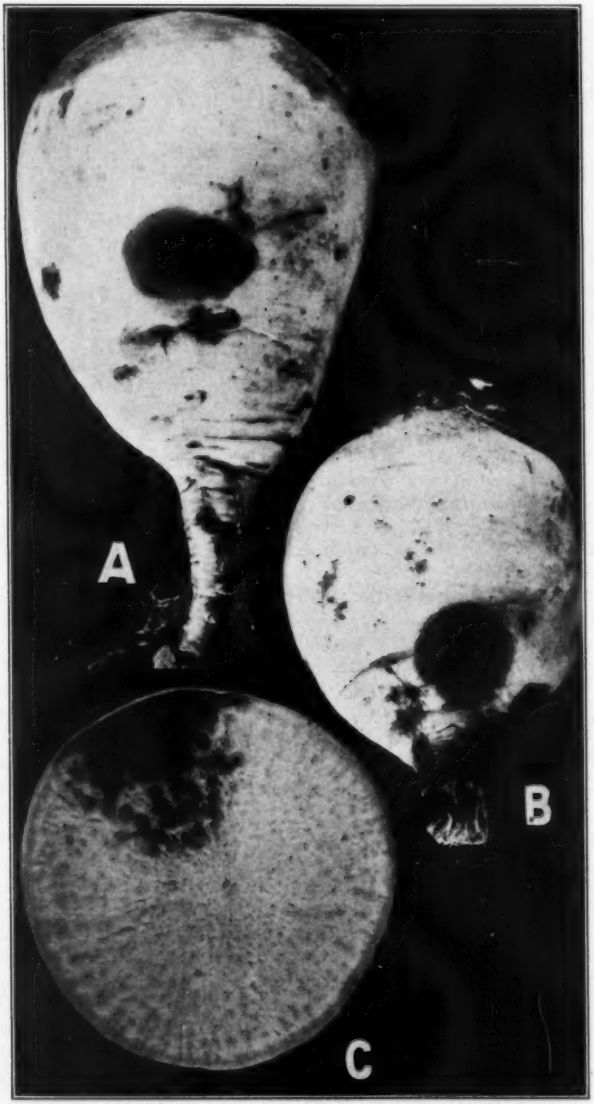

FIG. 1.—Rhizoctonia lesions on turnips: A and B, Surface views; C, cross section. × 0.9

Externally the lesions are grayish brown, often zonated with darker brown. (Fig. 1, A and B.) When the decay enters the purple areas of the roots, the purple in the necrotic tissue bleaches out. The color at the margin is often different from the normal purple, varying from pinkish red to dark purple. The mycelium and rusty-brown sclerotia may often be seen on the surface of the lesions.

MATERIAL, METHODS, AND EQUIPMENT

The fungi used in the experiments reported in this paper were Rhizoctonia (accession No. 167) isolated from turnip and three strains of *R. solani* (accession Nos. 334, 335, and 337) isolated from potato. The last three strains were obtained from Freeman Weiss, of the Bureau of Plant Industry. The cultures were free from other microorganisms, but were not single-hyphae strains.

Twelve varieties of turnips were used in these experiments. The Purple Top Globe variety was employed in the main storage and temperature experiments. The other 11 varieties were used in connection with a pathogenicity test with the Rhizoctonia from turnip. All the turnips were grown at the Arlington Experiment Farm and all were stored at a temperature of 0° to 2° C. except in cases otherwise specified in the context.

In the temperature and inoculation experiments the roots were washed in soapy water, rinsed in tap water, and the free water was dried off.

The roots were inoculated by inserting bits of mycelium, sclerotia, and agar from carrot-agar plates in the tissue by means of a sterile scalpel. The checks were merely punctured in the same way as the inoculated roots.

The temperature experiments were conducted in infection chambers described elsewhere (*5*). In the storage experiments the roots were stored in rooms 8 feet wide, 14 feet long, and 12 feet high.

PATHOGENICITY AND LIFE HISTORY OF THE CAUSAL ORGANISM

The turnip pathogene has been compared with three strains of *Rhizoctonia solani* isolated from potato and has been found to resemble them in its microscopic characters. It can, however, be distinguished from these strains in its gross characters on culture media. The outstanding character distinguishing it from the potato strains mentioned above is its tendency to produce sclerotia much more abundantly in culture.

The potato strains likewise can be distinguished from one another by their gross characteristics. This observation is in harmony with those made by other workers (*6*, *7*). The turnip pathogene is regarded as a strain of *Rhizoctonia solani*.

Two experiments were conducted to determine the relative ability of the three above-mentioned strains of *Rhizoctonia solani* from potatoes and the strain from turnips to infect turnips. The potato strains failed to produce infection, whereas the turnip strain infected 76 per cent of the roots inoculated. (Table 1.)

TABLE 1.—*Results of inoculating turnips with Rhizoctonia isolated from turnip and R. solani isolated from potato*

Accession No.	Host	Duration of experiment (days)	Number of roots inoculated	Number infected	Percentage infected
167	Turnip	8 and 12	29	22	76
334	Potato	8 and 12	29	0	0
335	do	8 and 12	22	0	0
337	do	8 and 12	29	0	0

The pathogenicity of strain No. 167 on turnip has been abundantly tested in connection with the temperature experiments given below, and positive results have been obtained in a high percentage of the cases. The following results, however, are submitted to meet the formal requirements of a test of pathogenicity: First, a Rhizoctonia has been found constantly associated with the disease during the last five years; second, the same fungus has been isolated from the same type of lesions consistently throughout this period; third, numerous inoculations with this organism have yielded positive results. The data given below are submitted as examples.

Fourteen inoculated roots and ten uninoculated ones were stored at a temperature of 10° C. for 21 days. By the end of this time all 14 inoculated turnips had become infected. Decay had just started in 3, and the remaining 11 showed lesions ranging from 10 to 40 mm. in diameter. Two of the ten uninoculated roots were infected; infection had just started in one of them, and the lesion in the other was about 30 mm. in diameter. The rest remained healthy. Eleven reisolations were made from the inoculated roots. Nine yielded Rhizoctonia, and two were sterile. Rhizoctonia was obtained from the larger lesion in the uninoculated roots.

In another experiment 19 turnips were inoculated with a culture obtained in the preceding experiment. Ten roots were punctured with a sterile needle and held as checks. Both uninoculated and inoculated roots were stored at a temperature of 10° C. After 45 days, 13 of the 19 roots were infected and showed typical lesions ranging from 15 to 50 mm. in diameter. Reisolations were made from 12 of the 13 lesions. Eleven of these yielded pure cultures of Rhizoctonia, and the twelfth yielded Rhizoctonia and bacteria. The check roots remained uninfected. It is believed that these results establish the pathogenicity of the organism.

The life history of the pathogene is imperfectly known, and what is said of it should be regarded as only suggestive.

Rhizoctonia is an organism that lives readily in the soil. Its presence there probably would be sufficient to account for it in storage. The mycelium and sclerotia could be easily carried into the storage house in the soil clinging to the roots. The observations made indicate that this is what happens. The disease has not been observed on the roots when they were placed in storage. At harvesting in the fall of 1925 a careful examination was made for lesions on the fleshy roots and infection of the secondary roots, but none was found. The amount of decay that occurred later in storage, taken in connection with the fact that disease was not evident for three or four weeks after storage, suggests that most of the infection took place after

the roots were stored. These observations, of course, do not preclude the possibility that some incipient infections that were not macroscopically evident may have been carried into the storage house or that in the cases in which a critical examination was not made at the time of storage the disease may have been present in roots when they were stored. The fact that the disease has been known to occur in the roots in the field (*3*) suggests that it is sometimes carried into storage.

After the pathogene is in the storage house it spreads during the storage period. The same strain has been observed to overrun carrots stored in the same room as the turnips, although it has not been seen on carrots when they were harvested. It rarely decays carrots but grows on them superficially. Only three carrots have been observed to be infected in storage during the seven years the writer has been carrying on storage experiments on carrots at the Arlington Experiment Farm.

METHOD OF INFECTION

Infection takes place for the most part through the tops, through the root tips, at points where the secondary roots emerge from the fleshy roots, and through wounds. Most of the infections occur through the secondary roots. It has not been determined whether or not infection can occur through the unbroken skin. Infection can occur so readily, however, through the openings mentioned above that it would seem that the additional ability to penetrate the skin would be of little importance in aggravating the disease.

SUSCEPTIBILITY OF VARIETIES

The following 12 varieties have been found to be susceptible to the disease: Navet à forcer demilong blanc, Navet du Limousin, Gros long d'Alsace, Jaune de Montmagny, Noir long, Noir rond, Petit de Belle Isle, Navet des Vertus Marteau, Purple Top Globe, Rose de Verdun, Rouge plat hâtif à feuille entière, and Rond des Vertus. Their relative susceptibility has not been determined. It may be said, however, that all the varieties are sufficiently susceptible to preclude marked resistance.

INFLUENCE OF TIME AND TEMPERATURE ON GROWTH OF PATHOGENE IN CULTURE MEDIA

In the following experiment the pathogene was grown in 200 c. c. Erlenmeyer flasks containing 40 c. c. of carrot agar. The flasks were inoculated by introducing bits of mycelium and agar, of as uniform size as possible, at the center of the medium of each flask. Ten flasks were placed at each temperature. The flasks were inspected at intervals and measurements made of the colonies when growth had occurred. The results are recorded in Table 2. No growth occurred at 34.5° C. in 29 days. The highest temperature at which growth took place was 31.5°. No growth occurred at 12° in 3 days, at 2° in 5 days, or at 0° in 29 days. The amount of growth decreased rapidly as the temperature rose or fell below 23°. The relation of temperature to growth is graphically represented in Figure 2. This experiment was supplemented by another in which the same relations were found. The minimum, optimum, and maximum temperatures for growth may be said to be approximately 0° to 2°, 23°, and 31.5° to 34.5°, respectively.

INFLUENCE OF TIME AND TEMPERATURE ON INFECTION AND DEVELOPMENT OF THE DISEASE

The results recorded in Table 3 were obtained from one among a number of temperature experiments, all of which were consistent in the temperature relations found. These data show more effectively, however, the influence of time on the infection and decay of the roots than do those not given, because they were taken more frequently and the periods are more nearly comparable in length.

Twenty inoculated roots were stored at each temperature, except in cases mentioned in the context, and measurements of the lesions were

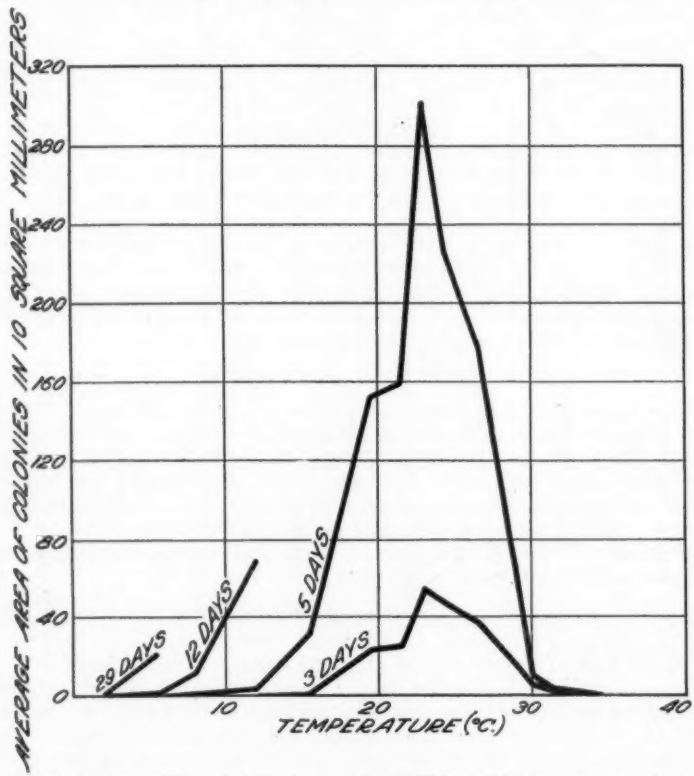

FIG. 2.—Influence of time and temperature on growth of Rhizoctonia from turnip on carrot agar

made after 1, 2, 3, 4, and 5 days at all the temperatures at which decay had occurred, and after 10, 16, and 29 days, depending on whether decay had occurred at the end of the various intervals at the lower temperatures or whether decay had advanced sufficiently to make measurements feasible. At the higher temperatures measurements were not made after more than five days, for two reasons: First, because decay had advanced to a point where the spherical surface of the lesion would have introduced considerable error if the

measurements had been continued, and, second, because Rhizopus had started to decay three roots at one of the temperatures (25° C.) on the fifth day. In this case the data used were based on 16 instead of 19 turnips.

TABLE 2.—*Influence of temperature and time on the growth of the turnip Rhizoctonia on carrot agar*

Temperature (°C.)	Average area of colonies in square millimeters after—			
	3 days	5 days	12 days	29 days
34.5	0	0	0	0
31.5	(a)	27		
30	45	101		
26.5	368	1,765		
24.5	466	2,273		
23	542	3,012		
21.5	247	1,590		
19.5	231	1,511		
15.5	3	300		
12	0	31	672	
8	0	5	110	
5.5	0	0.7	6	207
2	0	0	0.3	(a)
0	0	0	0	0

a Just started.

TABLE 3.—*Influence of time and temperature on the infection and decay of turnips inoculated with Rhizoctonia*

Temperature (°C.)	Number of roots used	Number of infections after the following days—								Average area of lesions in square millimeters after the following days—							
		1	2	3	4	5	10	16	29	1	2	3	4	5	10	16	29
32	20	0	0	0	0	0				0	0	0	0	0			
29	20	0	20	20	20	20				0	47	108	141	168			
25	19	8	19	19	19	19				3	74	138	205	a248			
23	20	5	20	20	20	20				2	68	169	250	336			
21.5	20	2	19	20	20	20				0.5	43	134	218	279			
19	16	0	15	16	16	16				0	22	88	156	255			
15.5	20	0	16	19	19	20				0	8	35	91	143			
10.5	20	0	1	5	11	17	20	20		0	0.2	1	9	23	160	442	
8	20	0	0	1	1	3	20	20		0	0	0.2	0.6	4	49	22	
5.5	20	0	0	0	0	2	13	17	19	0	0	0	0	0.9	14	81	319
2	20	0	0	0	0	0	2	3	10	0	0	0	0	0	0.3	1	9
1	20	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0.6

a This average was obtained from 16 lesions instead of 19, because 3 of the roots had started to decay with Rhizopus soft rot.

Time is a factor in determining both the number of infections and the extent of decay. At the higher temperatures the number of infections soon reaches a maximum. At the lower temperatures the maximum is reached more slowly. At 1° C. only one infection had occurred at the end of 29 days. This fact is of prime importance in limiting the amount of loss due to decay as well as the length of time

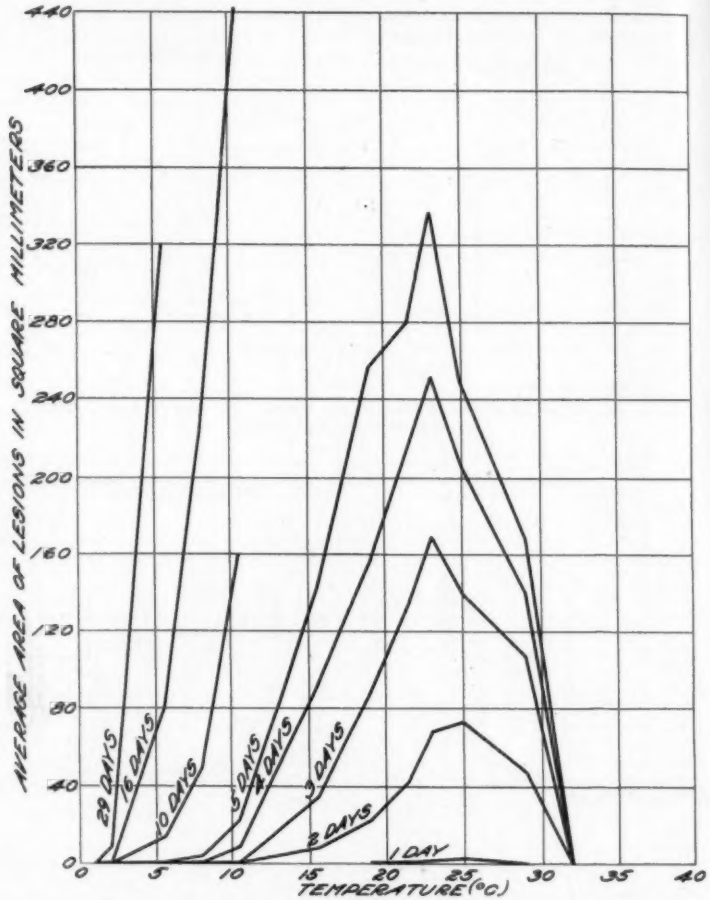

FIG. 3.—Influence of time and temperature on the infection and decay of turnips by Rhizoctonia

the roots may be stored. This conclusion is more clearly justified by the results obtained from some of the general storage experiments, to be discussed later, where infection depended upon the inoculum normally present in the roots and the penetration of the host by the fungus is not aided by artificial inoculation. (Table 6.) The effect of time on the degree of decay may be seen from an examination of Tables 3 and 4 and Figures 3, 4, and 5.

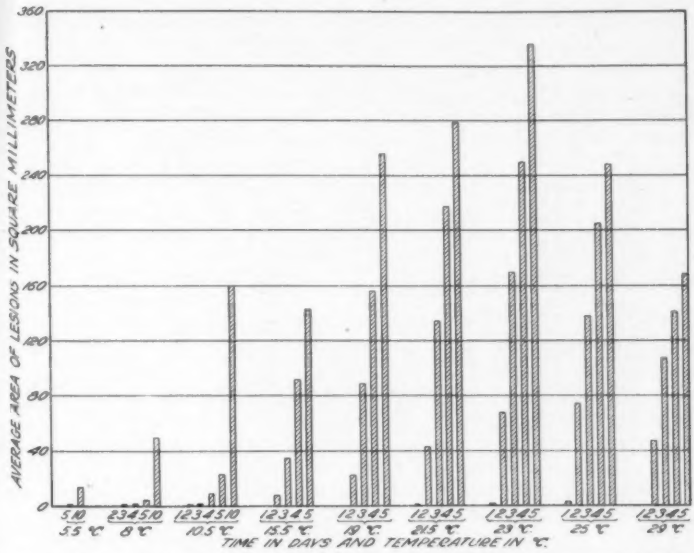

FIG. 4.—Influence of time and temperature on infection and the increase in area of Rhizoctonia lesions of turnips

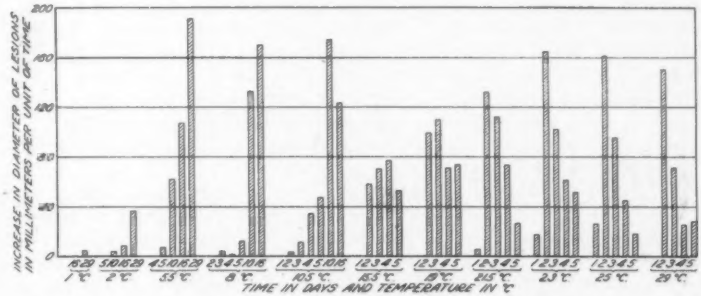

FIG. 5.—Influence of temperature on infection and increase in diameter of Rhizoctonia lesions per period of time

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TABLE 4.—*Influence of time on the increase in diameters of Rhizoctonia lesions on turnips stored at various temperatures*

Temperature (°C.)	Total diameters of 20 lesions and increase in diameter per period	Time in days							
		1	2	3	4	5	10	16	29
		Mm.	*Mm.*	*Mm.*	*Mm.*	*Mm.*	*Mm.*	*Mm.*	*Mm.*
32	Total diameter	0	0	0	0	0	------	------	------
	Increase in diameter	0	0	0	0	0	------	------	------
29	Total diameter	0	150	221	246	274	------	------	------
	Increase in diameter	0	150	71	25	28	------	------	------
25*	Total diameter	26	187	281	326	344	------	------	------
	Increase in diameter	26	161	94	45	18	------	------	------
23	Total diameter	17	182	284	345	396	------	------	------
	Increase in diameter	17	165	102	61	51	------	------	------
21.5	Total diameter	5	137	249	322	349	------	------	------
	Increase in diameter	5	132	112	73	27	------	------	------
19*	Total diameter	0	99	209	280	354	------	------	------
	Increase in diameter	0	99	110	71	74	------	------	------
15.5	Total diameter	0	58	128	205	258	------	------	------
	Increase in diameter	0	58	70	77	53	------	------	------
10.5	Total diameter	0	3	14	49	96	270	393	------
	Increase in diameter	0	3	11	35	47	174	123	------
8	Total diameter	0	0	3	4	16	149	319	------
	Increase in diameter	0	0	3	1	12	133	170	------
5.5	Total diameter	0	0	0	0	7	69	176	367
	Increase in diameter	0	0	0	0	7	62	107	191
2	Total diameter	0	0	0	0	0	3	11	48
	Increase in diameter	0	0	0	0	0	3	8	37
1	Total diameter	0	0	0	0	0	0	0	4
	Increase in diameter	0	0	0	0	0	0	0	4

* Only 16 and 19 roots were stored at temperatures of 19° and 25° C., respectively. In these cases the total diameter was computed by multiplying by 20 the average diameter of lesions calculated on the basis of the number of turnips used.

The data in Table 3 were compiled to show the effect of time and temperature on the extent of decay. The surface area of the lesion was selected as a standard of measurement because it more fairly represents the losses due to decay than do such standards as diameter. The volume or mass would, of course, give a more accurate picture than area, but not only are these standards difficult to apply in the present case but particular lesions can not be followed through the various periods of time.

Associated with such methods of measurement as surface area, volume, and mass is a factor affecting the rate of decay in addition to temperature and time. This factor is the amount of sound tissue exposed to the action of the fungus (area of reaction). This area of reaction increases progressively with and in proportion to the increase in the size of the lesions, which varies with temperature and the lapse of time.

One would expect, therefore, that after the pathogene had overcome the initial inertia involved in the process of infection the rate of decay at any given temperature would be in proportion to the increased area of reaction if the other factors remained constant. The data in Table 3 and the graphs in Figures 3 and 4 show some effect of this area-of-reaction factor at most of the temperatures and intervals of time, but the increase in rate with the passage of time is not in proportion to the increased area of reaction. This result seemed to point to an additional factor operating to slow up the rate of decay.

In order to isolate the slowing-up effect more definitely, it was decided to use the extension of the diameter of the lesions as a measure of the rate of decay, thus reducing the area of reaction to a constant.

The total diameter of 20 lesions and the increase in diameter for each period are given in Table 4, and extension of the diameter during each interval of time is plotted in Figure 5. When less than 20 turnips were used, as was the case at temperatures 25° and 19° C. (Table 4), the total diameter was computed by multiplying by 20 the average diameter of lesions calculated on the basis of the number of turnips used. The increase in diameter per unit of time was obtained by subtracting the total diameter at one period from the total diameter of the succeeding period in each instance.

An examination of the data in Table 4 and the graphs in Figure 5 shows—

(1) That very little or no decay took place during the first day at all temperatures.

(2) That at the lower temperatures there is an absence of decay for a longer period, and the lower the temperature the longer the period.

(3) That the rate of decay is relatively slow during the early stage of decay at all temperatures. (It is during these periods that the pathogene is establishing host relations. The process is obviously different from the process of decay that follows, and the difference in the effects of the two processes is clearly manifest in the results.)

(4) That the rate of extension of the diameter at temperatures of 10.5° to 29° C. increases after the initial period until it reaches a maximum and then declines.

(5) That at temperatures of 29°, 25°, 23°, and 21.5° C. the maximum rate is reached the second day, at 19° the third day, at 15.5° the fourth day, and at 10.5° the tenth day. Below 10.5° the rate is accelerated from the beginning of decay until the end of the experiment (29 days). This statement is intended to apply only to the periods of time employed in this experiment. Should these be altered, it is quite probable that the time required for the rate of decay to reach a maximum would likewise be altered at some of the temperatures, especially at 10.5° C., where a period longer than one day was involved in determining the maximum rate. Whether the rate of decay would have reached a maximum and then declined at these temperatures, had the time been extended, can only be conjectured.

All the periods of time except those of 16 and 29 days permit a direct comparison in rate. In the case of these last two periods there probably is some discrepancy in the values given, but it is not believed to be large enough to discredit the continued increase in rate until the end of the experiment at the lower temperatures.

What is the explanation of the decline in the rate of decay at the higher temperatures after a maximum had been reached? Attention should be called to the systematic error involved in the method of measuring the size of the lesions. The diameter was taken in two directions corresponding to the longest and the shortest diameters, which were nearly equal in each case. By this method of measurement there was a systematic shortening of the successive measurements, because the surfaces of the lesions were more or less spherical instead of flat. This error was small, however, judging by the average and maximum sizes of the lesions at the time the final measurements were made (Table 5), in connection with the sizes of the roots, which varied from 2.5 to 4 inches in diameter.

TABLE 5.—*Average and maximum diameters of Rhizoctonia lesions on turnips at the end of the storage period*

Temperature (°C.)	Average diameter of lesions	Maximum diameter of lesions	Duration of storage	Temperature (°C.)	Average diameter of lesions	Maximum diameter of lesions	Duration of storage
	Millimeters	*Millimeters*	*Days*		*Millimeters*	*Millimeters*	*Days*
29	14	29	5	10.5	20	32	16
25	17	28	5	8	16	25	16
23	20	36	5	5.5	18	32	29
21.5	17	27	5	2	2	7	29
19	18	25	5	1	0.2	4	29
15.5	13	22	5				

That this difference in successive measurements is not due to this error is further shown by the fact that the average as well as the maximum diameters of the lesions at 8° C. after 16 days and at 5.5° after 29 days are approximately the same as those obtained at the higher temperatures when the final measurements were made, and yet the rate of extension of the diameter of the lesions was increasing at the former temperatures rather than decreasing. This shift in rate is a part of a trend evident as one passes from a temperature of 23° (Table 4) to the lower temperatures for the maximum rate of increase as well as the decline in rate to be postponed as the temperature falls.

A factor presenting itself for consideration is what Brown (*2*) has chosen to call staling. He found a retardation in the rate of growth after it had reached a maximum in the case of certain fungi under some culture conditions due to the accumulation of toxic substances in the culture media.

It seems reasonable to believe that in the present case some toxic substance diffuses from the decayed area into the sound tissue, and that as the decay advances this substance accumulates at the borders of the lesions, tending more or less to check the advance of the fungus. Since these results were incidental to the main purpose of the experiments, no opportunity was afforded either to determine the nature of this retarding effect or to identify it with a particular substance.

The range of temperature at which infection and decay will occur extends from 0.5°, and possibly lower, to 29° C. (Tables 3, 4, and 7.) No infection occurred at 32° in five days, showing that 29° is near the maximum temperature for infection. The optimum temperature for infection and decay ranges from 19° to 25°. (Tables 3 and 4; figs. 3 and 4.) The amount of decay increases rather rapidly as the temperature rises from 1° to 23° and 25°, and then it sharply declines to zero at 32°. The infection-decay curves (figs. 3 and 4) correspond closely with growth curves of the pathogene on artificial media (fig. 2) in their cardinal points.

DEVELOPMENT OF DECAY UNDER CERTAIN STORAGE CONDITIONS

During the storage season of 1924–25 roots of the Purple Top Globe variety of turnips were stored at four different temperatures (10°, 8°, 4°, and 1° C.) with the relative humidity varying somewhat. At the end of the storage season (125 days) a count was made of the number of roots infected and not infected by Rhizoctonia in five

crates at each temperature. This count was based on what seemed to be the same disease throughout, the causal factor being then unknown. Out of 54 isolations on carrot-agar plates, 47, or 87 per cent, yielded Rhizoctonia, 4 a species of Mucor, 1 an unidentified fungus, and 2 plates were sterile. It is believed, therefore, that the losses recorded (Table 6) for this season were due primarily to Rhizoctonia.

TABLE 6.—*Infection of turnips by Rhizoctonia at different storage temperatures and relative humidities during the seasons of 1924–25 and 1925–26*

Storage season	Duration of storage (days)	Temperature (°C.)	Relative humidity (per cent)	Number of crates used	Number of turnips used	Number of turnips infected	Percentage infected
1924–25	125	10	80 to 95	5	641	397	62.0
		8	80 to 95	5	770	523	68.0
		4	80 to 85	5	669	3	.4
		1	90 to 95	5	506	4	.8
1925–26	100	10	80 to 95	2	389	340	87.0
		8	80 to 95	2	434	294	68.0
		1 to 2	88 to 93	4	884	17	2.0

Sixty-two per cent of the roots were infected at 10° C., 68 per cent at 8°, and 0.4 and 0.8 per cent at 4° and 1°, respectively. Although temperature is not the only factor determining the number of infections, it appears to be the predominating one. Humidity is also a factor, as will appear more definitely below. There were more infections at 8° than at 10°, and more at 1° than at 4°, where the humidities were higher in each case, if the saturation deficit is used as a measure of humidity.

In the storage season of 1925–26 the turnips were stored at three temperatures (10°, 8°, and 1° to 2° C.). Eighty-seven per cent (Table 6) were infected at 10°, 68 per cent at 8°, and 2 per cent at 1° to 2° after 100 days of storage. Eighty-four isolations were made to carrot-agar plates from lesions on roots from the three storage conditions, with the following results: 62 plates, or 74 per cent, yielded Rhizoctonia; 1, Alternaria; 7, bacteria; 1, Fusarium; 4, unidentified fungi; and 9 were sterile.

Another experiment was conducted during the season 1925–26, in which, in addition to the control of temperature, the humidity was more accurately controlled. Fifteen roots were stored at each of nine combinations of temperature and humidity—three temperatures, 10°, 4.5°, and 0.5° C., and three humidities at each temperature. At 10° the relative humidities were 61, 74, and 95 per cent; at 4.5°, 70, 74, and 96 per cent; and at 0.5°, 80, 85, and 96 per cent. There were no infections at any of the humidities, except the highest at each temperature. The results obtained at the highest humidity at each temperature are to be found in Table 7. The percentage of infection at 10° was 80; at 4.5°, 53; and at 0.5°, 7 per cent at the end of 82 days of storage.

These results indicate that the most effective method of eliminating Rhizoctonia rot would be to store turnips at a low humidity, but the effects of low humidity on the roots are such as to preclude the use of all the humidities employed in this experiment except the highest at each temperature. The roots stored at the two lower humidities at each temperature were badly shriveled and those at the two higher temperatures were almost mummified.

TABLE 7.—*Influence of temperature at practically constant relative humidities on the infection of turnips by Rhizoctonia and resultant decay after 82 days of storage*

Temperature (°C.)	Relative humidity (per cent)	Number of turnips used	Number infected	Percentage infected	Total number of lesions	Average area of lesions (square millimeters)	Total area of lesions (square millimeters)
10	95	15	12	80	61	431	26,272
4.5	96	15	8	53	10	419	4,187
0.5	96	15	1	7	1	147	147

The effect of temperature at the higher humidities, on the other hand, is such as to make it practicable to store turnips at or near 0° C. for long periods without incurring much loss. This fact is shown not only by the low percentage of infection but by the degree of decay measured by the average and total areas of the lesions at this temperature as compared with those obtained at the higher temperatures.

CONTROL MEASURES

The results recorded in Tables 3, 6, and 7 show that Rhizoctonia rot of turnips can be effectively controlled by storing the roots at or near 0° C., at least for a period of three to four months or more from the time they are harvested. The data on the humidity relations are incomplete, but from those that are available it is believed that the number of infections could be limited by reducing the relative humidity at from 0° to 2° C. to about 90 per cent, and still maintain a desirable turgor of the roots.

SUMMARY AND CONCLUSIONS

A disease of turnips caused by Rhizoctonia has occurred in the stored crop grown at the Arlington Experiment Farm during the last five seasons and has been observed occasionally in both turnips and rutabagas at the terminal markets.

A description of the disease and an illustration showing surface and cross-section views of the lesions are given.

The pathogene resembles *Rhizoctonia solani* from potato in its microscopical characters and is believed to be a strain of this species. It can be distinguished from strains isolated from potatoes by its abundant production of sclerotia on culture media. The tested potato strains can be distinguished from each other by their gross characters on culture media. The potato strains were not able to infect turnips.

It is not known that the disease in storage is contingent upon field infection, though the latter has been reported to occur. Turnips apparently free from infection of either the fleshy or secondary roots when harvested become infected during storage. The presence of the causal fungus in the soil that clings to the roots is sufficient to account for the disease in storage. Sound roots readily become infected under favorable conditions, infection taking place largely through the root tips, tops, and secondary roots.

Twelve varieties of turnips have been found susceptible to the disease.

The pathogene has been observed to grow at a range of temperatures from 2° to 31.5° C. (Table 2.) Growth had not occurred at 34.5° by the end of 29 days, and it had merely begun at 2° in the same time. The maximum growth took place at 23°. The maximum, minimum, and optimum temperatures for growth within the time limits of the experiment reported here may be said to be 31.5° to 34.5°, 0° to 2°, and about 23°, respectively.

The temperature range for infection, as represented by the temperatures employed, extends from 0.5° to 29° C. (Tables 3 and 7.) One infection out of 20 inoculations occurred at 1° (Table 3) in 29 days, and 1 infection in 15 uninoculated roots at 0.5° after 82 days (Table 7). Twenty infections out of 20 inoculations took place at 29° in 2 days, but no infections occurred at 32° in 5 days. The greatest amount of decay occurred at temperatures ranging from 19° to 25°. The maximum, optimum, and minimum for infection and decay may be said to be represented by 29° to 32°, 19° to 25°, and somewhere near 0°, respectively.

The maximum number of infections in the inoculation experiment was obtained by the end of the second day at 29°, 25°, and 23° C.; the third day at 21.5° and 19°; the fifth day at 15.5°; the tenth day at 10.5° and 8°. The maximum number of infections may have occurred between the fifth and the tenth days at 10.5° C., but no data were taken during this period. The maximum number possible (Table 3) was never obtained at temperatures below 8°, the number dropping off rapidly with the lowering of the temperature. This fact is important in limiting the percentage and amount of decay at temperatures near 0°.

The rate of extension of the diameter of the lesions is relatively slow during the period immediately following inoculation. (Tables 3 and 4; figs. 3, 4, and 5.) The slowness of rate may be explained on the basis that decay does not begin immediately after inoculation, some time being required for infection to occur. After this period the rate of decay increases more or less rapidly, depending upon the temperature at which the roots are stored. (Table 4.) Within the time limits given in Tables 3 and 4 the rate reaches a maximum by the end of the second day at temperatures of 29°, 25°, 23°, and 21.5°; by the end of the third day at 19°, by the end of the fourth day at 15.5°, and by the end of the tenth day at 10.5° C., and then declines at all these temperatures. At 8° the rate increases until the last measurements are made (16 days); at 5.5° and 2° it continues until the end of the experiment (29 days). Decay had begun in one root at 1° in 29 days.

The retardation in the rate of extension of the diameter of the lesions at the higher temperatures is similar to that observed by Brown (*2*) in some fungi in culture media. The cause of the retarding effect is not known.

The rate of decay as the temperature rises from 1° to 23° and 25° C. is rapid and drops off rather suddenly as the temperature rises above 25°, until at 32° there is no decay. (Table 4; figs. 3 and 4.)

The losses due to Rhizoctonia rot of turnips depend largely upon the conditions of storage, the most important of which are humidity

and temperature. High humidity favors infection and decay and low humidity limits both. Humidity as a controlling factor has its limitations, however, because of the tendency of the roots to shrivel due to loss of water at humidities below 85 per cent at any of the temperatures employed.

Temperature is the most important factor in the control of the disease. It is possible to store roots at a temperature ranging from 0° to 2° C. for three or four months or more without incurring much loss. As the temperature is raised to 4° the amount of decay increases rapidly. The losses in storage during two years at temperatures of 8° to 10° ranged from 62 to 87 per cent. (Table 6.)

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THE CORRELATION BETWEEN THE SOIL SALINITY AND FLOWERING DATE IN COTTON[1]

By J. Arthur Harris

Head, Department of Botany, University of Minnesota, and Collaborator, Office of Alkali and Drought Resistant Crops, United States Department of Agriculture

INTRODUCTION

In an earlier paper (*7*)[2] evidence has been adduced to show that under certain conditions there is a positive correlation between soil salinity and seedling stand in cotton. On certain experimental plots on the saline soils of the Gila River Valley at the United States Field Station, Sacaton, Ariz., larger numbers of seedlings were produced on the more saline areas of the experimental field.

In the paper cited it was pointed out that such relationships could not be expected over an indefinitely wide range of soil salinity. Notwithstanding these cautions and the suggestions made concerning the explanation of the results for seedling stand, it is possible that these findings may be interpreted by those who are not acquainted with the full complexity of the problem as indicating that under irrigation in the Southwest highly saline soils are actually better than those of low salinity for cotton production.

Such conclusions might seem to be supported by the fact that, as related by Kearney and Means (*14*) and by Kearney (*13*), cotton is one of the most salt-resistant of the staple crops. Balls (*1*, *2*) made observations that lead to the same conclusions. Finally, detailed investigations made for the Office of Alkali and Drought Resistant Crops of the Bureau of Plant Industry have shown not only that the chloride (*12*) and sulphate (*9*) contents of the leaf-tissue fluids of cotton are high, but also that there is a differential absorption of these anions (*4*, *6*, *10*, *11*).

Within certain ranges of soil salinity a positive correlation between salinity and seedling stand has been found (*7*). In view of this fact it is desirable to extend investigations to the consideration of the relationship between the properties of the soil and other characteristics of the plant for the purpose of determining whether, within certain rather narrow and tolerable ranges of soil salinity, higher values of soil salinity have a beneficial or a deleterious influence on the plant.

An elaborate investigation has been made of the relationship between the electrical conductivity of the soil on the one hand and the osmotic concentration (as expressed in terms of freezing-point depression), specific electrical conductivity, and chloride content of

[1] Received for publication Oct. 29, 1928; issued February, 1929.

[2] Reference is made by number (italic) to "Literature cited," p. 112.

the plant-tissue fluids on the other (*5*). For present purposes, however, a series of investigations of the relationship between the salinity of the soil and some functional characteristics of the plants grown, such as time of maturity or crop yield or quality, is needed.

The purpose of this paper is to consider the relationship between soil salinity and the time required for the plants to reach the stage of development indicated by the production of the first flower.

MATERIALS AND METHODS

The cultures considered are those of an experiment made in 1922. This set of cultures has furnished data on the chloride content (*12*) of the tissue fluids of Pima Egyptian and Meade and Acala upland cottons. The arrangement of the cultures has been fully described in papers on the relationship between soil salinity and the physico-chemical properties of the leaf-tissue fluids (*5*) and between soil salinity and seedling stand (*7*). These papers, as well as one on the regression of the soil properties of neighboring plots (*8*), may be consulted for details of arrangement of plantings.

By means of the soil bridge (*3*), soil-resistance determinations were made on samples from the 72 subplots devoted to each of the three varieties. These subplots were arranged in sequence so that the three varieties were uniformly distributed over the entire experimental area, which was 180 by 79.5 feet in dimension. Each subplot comprised two rows each of 10 hills 1 foot apart. Three soil borings were made at distances separated by about 3.3 feet in each subplot, but the cores were combined in order to give an average sample for the plot as a whole.

The order of magnitudes of the soil resistances have been indicated elsewhere (*7*).

Since each soil determination serves for a number of plants, the soil-conductivity determinations were weighted with the number of plants produced on each subplot in the determination of the means and standard deviations of soil resistance and in the determination of the correlations between soil resistance and flowering date.

Since soil borings were made in three series, each of 72 subplots containing originally 20 hills of cotton, a total of 1,440 plants of each variety would have been available if every hill had produced one plant. This result can not generally be realized. All available plants were considered. The date of flowering was determined for 870 plants of Pima Egyptian cotton, for 725 plants of Meade upland cotton, and for 999 plants of Acala upland cotton. Since the present paper is concerned primarily with the correlation between the soil properties and the flowering date rather than with the question of differences in earliness of maturity of the different varieties, it seems unnecessary to deal specifically with the means, standard deviations, and other constants for the time of flowering.

RESULTS

The correlation coefficients measuring the relationships between the electrical resistance of the soil and the time required by the plant to reach the flowering stage are given in Table 1. Coefficients

for the resistance of each of the four soil layers of 1 foot each, R_1 to R_4, are given. The average values are the mean of these four coefficients. The correlation between the average resistance of the four individual layers, R_{1-4}, and the period required for flowering are also included.

Since soil resistance is measured in ohms (high resistance indicating low salinity and vice versa) and the time of flowering is measured in number of days after a given origin date for the determination of flowering records, a negative correlation between the two variables shows that later flowering is associated with lower values of the electrical resistance of the saturated soil mass, or with higher salt content.

TABLE 1.—*Correlation coefficients measuring the relationship between soil salinity (in terms of electrical resistance) and time of flowering in Egyptian and upland cottons grown at the United States Field Station, Sacaton, Ariz., in 1922*

Depth of soil sample	Pima (Egyptian)		Meade (upland)		Acala (upland)	
	$r \pm E_r$	r/E_r	$r \pm E_r$	r/E_r	$r \pm E_r$	r/E_r
First foot, R_1	−0.1097±0.0226	4.85	−0.0902±0.0248	3.63	−0.0182±0.0213	0.85
Second foot, R_2	−.1535±.0223	6.87	−.1198±.0247	4.85	−.0841±.0212	3.97
Third foot, R_3	−.1075±.0226	4.76	−.1031±.0248	4.16	−.0486±.0213	2.28
Fourth foot, R_4	−.0723±.0228	3.18	−.1006±.0248	4.06	−.0678±.0212	3.19
Average correlation	−.1108		−.1034		−.0547	
First to fourth foot, R_{1-4}	−.1288±.0225	5.73	−.1111±.0247	4.49	−.0507±.0213	2.38

The coefficients are all negative. While in general the values are low, a number of the constants may be considered significant in comparison with their individual probable errors. The average correlations for the resistance of the first to the fourth foot are −0.111 for Pima Egyptian, −0.103 for Meade upland, and −0.055 for Acala upland cotton. These may be compared with the correlations between the average resistance of the first to the fourth foot R_{1-4}, which are −0.129 for Pima Egyptian, −0.111 for Meade upland, and −0.051 for Acala upland cotton.

Whether the generally lower correlation for Acala cotton is significant, and possibly related to the earlier flowering of this variety, must be determined by further investigation.

SUMMARY

Consideration of the correlations between soil resistance and flowering date in an extensive culture of Pima Egyptian, Meade upland, and Acala upland cottons, made at the United States Field Station in the Gila River Valley at Sacaton, Ariz., indicates that there is a low negative correlation between the soil resistance and the time required for flowering in these three varieties of cotton. Since soil salinity is measured in terms of resistance, while flowering date is measured in terms of days after an arbitrary origin date (June 1), negative correlation coefficients indicate that the flowering date is slightly retarded by soil salinity.

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PHOSPHORUS DEFICIENCY IN FORAGE FEEDS OF RANGE CATTLE[1]

By SAMUEL G. SCOTT

Assistant Chemist, Montana Agricultural Experiment Station

INTRODUCTION

The bone-chewing habit of cattle, apparently due to some mineral deficiency in their feed, has been under observation for a number of years in certain sections of Montana. It has recently been recognized that these symptoms affect cattle over very large areas of grazing land, and that the condition is becoming more acute as the better class of land is reserved for farming purposes, thus limiting the grazing land to the areas with poorer soil.

Reports received by the veterinary department of the Montana Experiment Station of cattle exhibiting depraved or perverted appetite indicate that the disorder is of such a serious nature as to interfere materially with the livestock industry in Montana in such affected areas. Consequently an investigation to determine the cause of the trouble was begun in 1923 by the veterinary and chemistry departments.

REVIEW OF LITERATURE

Mineral deficiencies in the ration of cattle have been reported from many localities in the United States. Welch (*21*)[2] calls attention to the fact that as long ago as 1910 the veterinary department of the Montana Experiment Station investigated cases of this kind, and made recommendations for relief. Huffman and Taylor (*13*) state that depraved appetite in dairy cattle is fairly common in certain sections of Michigan. Eckles and associates (*4*) report that the Minnesota Experiment Station has for several years been cognizant of a dietary deficiency of cattle in western Minnesota. Schmidt (*18*) tells of a rapidly fatal disease of cattle occurring on the prairie region along the Gulf of Mexico in Texas and known to cattlemen in that territory for a long time. Hart and coworkers (*8*) state that for 10 years or longer a "mysterious disease" has been observed among some of the dairy herds of Wisconsin. References to the occurrence of the trouble in a number of other States are to be found in various agricultural publications.

Nutritional disorders of cattle and other animals seem to occur more or less all over the world. They have been reported from Australia (*7*, *11*), Norway (*20*), New Zealand (*2*, *3*, *16*, *17*), Africa (*19*), and Germany (*15*). No attempt will be made here to give a complete review of the literature published. The extensive work of Theiler and associates (*19*) shows that phosphorus deficiency is a general characteristic of a very large proportion of the total acreage of South Africa, producing serious difficulties in raising cattle on the

[1] Received for publication Nov. 9, 1928, issued February, 1929.

[2] Reference is made by number (italic) to "Literature cited," p. 129.

veldt as the result of low mineral content in forage grasses. König and Karst (*15*) in their rather complete report mention the prevalence of mineral deficiency disorders among cattle in several localities in Germany, and refer to similar conditions in parts of Switzerland, Austria, and Hungary.

METHODS OF EXPERIMENTATION

In 1925 a study was undertaken by the veterinary department of the Montana Experiment Station to ascertain whether a deficiency of calcium and phosphorus existed in the grasses and forage crops of Montana used as feed for livestock. Areas where either osteomalacia or bone-chewing habits had been reported as being quite prevalent among cattle were selected by the veterinary department for this investigation. Samples of forage grasses, hay, water, and soil were collected from these areas, over a period of three years. Samples of forage were also collected for purposes of comparison from areas where cattle did not show the bone-chewing symptoms, and where mineral deficiency disorders were not prevalent.

LOCATIONS OF INVESTIGATED AREAS

The preliminary work done the first year on the study of mineral deficiencies was confined to certain sections of the Gallatin Valley, near Manhattan, in Gallatin County, and near Waterloo, in Madison County. Farmers in both of these areas had reported cattle with depraved or perverted appetite, manifested by the eating of materials, including bone, which could not ordinarily be classified as food. Quite generally this abnormal appetite was most pronounced in the spring, following limited winter feeding.

A few additional samples of forage grasses were submitted at the beginning of the investigation by county agents and farmers from other affected areas in the State, but the samples collected by members of the chemistry department are the ones used from which the results recorded herein were obtained.

In 1925, because of an inclement growing season, soil samples were not collected at the same time as were forage samples. However, in 1926, when additional samples of forage were again collected in Gallatin and Madison Counties, soil samples were also taken in the affected areas.

Possibly the most severe conditions studied in this work prevail in Teton County. Range cattle in affected areas in this territory are entirely dependent on wild grasses and wild hay for their food supply. A crop of hay that supplies the winter fodder is gathered from meadows or pastures adjoining those which furnish the summer feed. Samples of grasses, soil, and water were collected from several affected areas in Teton County, near Choteau, during the growing season of 1926.

In order to determine whether the influence of moisture during the growth of forage plants had any effect on their calcium and phosphorus content, samples of forage grasses were again collected in 1927, following a wet growing season, in Gallatin and Teton Counties. These samples were collected to duplicate, as nearly as possible, samples previously taken in these two counties in 1925 and 1926.

ANALYTICAL PROCEDURE

All samples collected during this study of mineral deficiencies were analyzed according to the methods adopted by the Association of Official Agricultural Chemists (*1*). The forage samples were dried and ground and moisture determined on the air-dried samples. These samples were then incinerated in an electric muffle at a dull red heat, and calcium and magnesium determinations were made on the resulting ash. Phosphorus determinations were made on the ash of the ground samples, which had been treated with magnesium nitrate before incineration to prevent any possible volatilization of organic phosphates. Calcium, magnesium, and phosphorus determinations on the soil and water samples were made in the usual manner, the soil samples having also been treated with magnesium nitrate before incineration. The analytical data on all forage samples are calculated and reported on the dry basis.

EXPERIMENTAL DATA

ANALYSES OF FORAGE PLANTS

The results of analyses of the different forage samples collected in the survey of the various areas are given in Tables 1 to 5 inclusive.

TABLE 1.—*Average percentages of calcium and phosphorus in forage-plant samples in an unaffected area in Gallatin County, Mont., 1925 and 1927*

[The 1925 samples were collected in July; the 1927 samples in August]

Forage plant	Samples analyzed	Year	Calcium (CaO)	Phosphorus (P_2O_5)
	Number		*Per cent*	*Per cent*
Timothy	13	1925	0.289	0.380
Do	10	1927	.293	.254
Alfalfa	11	1925	2.01	.415
Do	9	1927	2.15	.376
Clover	3	1925	1.47	.369
Do	2	1927	1.72	.282
Redtop	3	1925	.314	.345
Do	4	1927	.425	.321
Bunch grass	9	1925	.345	.373
Do	7	1927	.274	.274

TABLE 2.—*Percentages of calcium and phosphorus in hay samples from affected areas near Waterloo, in Madison County, Mont., July 2, 1925*

[All samples were taken from hay stacks]

Kind of hay	Calcium (CaO)	Phosphorus (P_2O_5)	Kind of hay	Calcium (CaO)	Phosphorus (P_2O_5)
	Per cent	*Per cent*		*Per cent*	*Per cent*
Alfalfa	3.01	0.206	Alfalfa	2.91	0.297
Do	2.06	.222	Do	2.04	.254
Do	1.88	.185			
Do	3.67	.215	Average	2.39	.249
Do	2.23	.300			
Do	1.49	.268	Alfalfa and timothy	1.69	.284
Do	2.21	.293	Alfalfa and clover	1.78	.359

TABLE 3.—*Percentages of calcium and phosphorus in freshly cut alfalfa of the first crop from affected areas near Waterloo, Madison County, Mont., June 2, 1926*

[Same areas as listed in Table 2]

Kind of sample	Calcium (CaO)	Phosphorus (P_2O_5)	Kind of sample	Calcium (CaO)	Phosphorus (P_2O_5)
	Per cent	*Per cent*		*Per cent*	*Per cent*
Alfalfa	3.15	0.403	Alfalfa	3.14	0.337
Do	3.48	.419	Do	2.66	.335
Do	3.67	.350			
Do	3.39	.371	Average	3.25	.369

TABLE 4.—*Average percentages of calcium and phosphorus in forage-plant samples from a ranch in an affected area near Manhattan, Gallatin County, Mont., 1926 and 1927*

[The 1926 samples were collected in June, the 1927 samples in July]

Forage plant	Samples analyzed	Year	Calcium (CaO)	Phosphorus (P_2O_5)
	Number		*Per cent*	*Per cent*
Alfalfa	3	1926	2.82	0.367
Do	1	1927	2.09	.324
Clover	1	1926	2.55	.337
Do	1	1927	2.04	.318
Wild grass (prairie rush grass and juncus)	3	1926	.466	.213
Do	2	1927	.430	.128

TABLE 5.—*Comparison of percentages of calcium and phosphorus in wild-grass samples from five different ranches in affected areas near Choteau, Teton County, Mont., 1926 and 1927*

Type of ranch	Analysis of 1926 samples		Analysis of 1927 samples	
	Calcium (CaO)	Phosphorus (P_2O_5)	Calcium (CaO)	Phosphorus (P_2O_5)
	Per cent	*Per cent*	*Per cent*	*Per cent*
Wet lowland	0.31	0.237	0.39	0.198
Do	.70	.133	.77	.082
Do	.53	.184	.59	.119
At higher elevation	.31	.275	.36	.134
Do	.36	.282	.30	.182

TABLE 6.—*Summary of average mineral composition of forage from unaffected and affected areas in Montana*

Forage plant and locality	Samples analyzed	Calcium (CaO)	Phosphorus (P_2O_5)	Magnesium (MgO)
	Number	*Per cent*	*Per cent*	*Per cent*
Alfalfa:				
Unaffected areas	10	2.02	0.423	0.479
Affected areas	14	2.59	.284	.533
Clover:				
Unaffected areas	3	1.47	.356	.722
Affected areas	4	2.27	.299	.716
Wild grasses:				
Unaffected areas	9	.341	.360	.119
Affected areas	14	.441	.259	.275

Table 1 gives the average per cent of calcium and phosphorus, expressed as oxides, found in different types of forage grasses collected at various locations covering most of the Gallatin Valley, and is representative of the forage fed to stock in Gallatin County. The samples included in this table were taken from locations where bone-chewing cattle have never as yet been reported. A comparison of the percentages of calcium and phosphorus in the different types of forage for the years 1925 and 1927 is given. The samples taken in 1925 were collected shortly after the middle of July; the corresponding samples taken in 1927 were collected about the middle of August. Because of a wet spring in 1927, especially during the month of May, the growing season for 1925 was three or four weeks in advance of that of 1927. Hence the cutting of samples for both years was made at practically the same stage of growth.

Table 2 gives the percentages of calcium and phosphorus found in hay samples collected from areas near Waterloo, in Madison County, the early part of July, 1925. The samples listed in this table were gathered from hay stacks, at least 1 year old. Bone chewing by cattle has been reported from areas in this district, even though legume hays are fed to stock in abundance. An inspection of the analyses in Table 2 shows that the alfalfa hay is adequately supplied with lime, but apparently contains less than the normal amount of phosphorus found in good hay. Nutritional disorders with cattle in these areas are probably the result of a phosphorus deficiency in the forage.

Alfalfa samples were collected in affected areas in Madison County, June 2, 1926, previous to the first cutting. Analyses of these samples are given in Table 3, and represent the same type of forage as listed under alfalfa hay in Table 2. The differences between the lime and phosphorus content of the newly cut alfalfa and that contained in the stacks would indicate that some plant minerals have been lost.

Table 4 gives the calcium and phosphorus content of alfalfa, clover, and wild-grass samples collected in 1926 and 1927 from a ranch near Manhattan, in Gallatin County, where the bone-chewing habit is prevalent.

Table 5 shows the comparative percentages of calcium and phosphorus found in wild-grass samples collected at five different locations in Teton County in 1926 and 1927. The samples collected from the first three ranches listed were grown on wet, lowland areas. The other two samples were collected at somewhat higher elevations.

Table 6 is a summary of the analyses of a number of samples of alfalfa, clover, and wild grass. The analyses are divided into two groups, "unaffected" and "affected," and are so named upon the information received about the areas at the time of collecting the samples. Magnesium determinations of the forage samples are included in this table, since it was thought that magnesium might be a factor in the study of mineral deficiency.

WILD-GRASS VARIETIES

Pure stands of grasses do not occur in the areas where samples listed under Tables 4, 5, and 6 were collected. The wild-grass samples from near Manhattan, in Gallatin County, on an affected area (Table 4) were identified by the station botanist as mostly juncus

and prairie rush grass (*Muhlenbergia cuspidata*). The grasses from affected areas in Teton County, near Choteau (Table 5), were identified as mixtures of squirrel tail (Hordeum), redtop (Agrostis), sedge (Carex), slough grass (Beckmania), and juncus in the lowland areas and bluestem (Agropyron), timothy, upland bunch grasses (Calamagrostis and Calamovilfa), and wheatgrasses (*Agropyron tenerum*) in the upland areas.

ANALYSES OF SOIL FROM AFFECTED AREAS

Table 7 gives the analyses of calcium, phosphorus, and magnesium in soil samples, to the depth of 1 foot, taken in affected areas. All samples show high lime content, and are fairly well supplied with phosphorus. These soils are probably all of dolomitic origin, with considerable variation in the calcium and magnesium ratio. The samples of soil from Madison County, near Waterloo, represent bench land soil of lime formation. The samples collected in Gallatin County, near Manhattan, are also "high lime soils," especially one sample analyzing 20.40 per cent CaO, which was taken from a grazing area used as a summer pasture. These samples represent soil from lowland areas bordering on alkali flats.

TABLE 7.—*Calcium, phosphorus, and magnesium in soil samples from affected areas in Montana, 1926*

Sample No.	Location	Date of sampling	Calcium (CaO)	Phosphorus (P_2O_5)	Magnesium (MgO)
			Per cent	*Per cent*	*Per cent*
1	Madison County	June 2	4.60	0.142	0.641
2	do	do	2.35	.128	.357
3	do	do	5.25	.101	.320
4	do	do	3.15	.101	.626
5	do	do	3.05	.137	.266
6	do	do	3.65	.133	.451
Average			3.67	.123	.443
7	Gallatin County	June 4	6.60	.160	.543
8	do	do	4.55	.160	.314
9	do	do	5.30	.147	.387
Average			5.48	.155	.414
10 [1]	Gallatin County	June 4	20.40	.141	3.555
11	Teton County	June 7	25.70	.188	1.50
12	do	do	30.75	.133	1.34
13	do	do	27.90	.147	.941
14	do	do	17.05	.153	.724
Average			25.35	.155	1.126
15 [2]	Teton County	June 7	2.30	.167	.271

[1] Summer pasture. [2] Upland area.

Samples collected in Teton County show a much higher lime content, with the exception of one sample, than do those from any of the other areas where soil samples were collected. These soil samples with the one exception, were all collected from wet lowland areas. One sample, the exception, was taken at a higher elevation.

The bone-chewing habit of cattle has been reported as most prevalent in areas where the stock graze during the summer on the forage

growing on low, wet land. The phosphorus content of all soil samples in Table 7 indicates an adequate supply of this mineral. But the analyses of forage grown on such soil also indicates that the phosphorus is not available in sufficient amounts as plant food when needed.

ANALYSES OF WATER FROM AFFECTED AREAS

Samples of water were collected from different creeks in two of the affected areas. These creeks are the source of the water supply for the range cattle in these areas. Table 8 gives the analyses of water used at five different ranches. Samples 1, 2, 3, and 4 represent creek water from four ranches in Teton County. Sample 5 was collected from a creek passing through an affected ranch near Manhattan in Gallatin County. None of these samples showed any appreciable amount of phosphorus on analysis, and the calcium content of each sample was from two to three times greater than the magnesium.

TABLE 8.—*Amounts of calcium, phosphorus, and magnesium in creek water in two affected areas in Montana*

Sample No.	Parts per million of—			Calcium magnesium ratio
	Ca	P	Mg	
1	55	Trace	25	2.2 : 1
2	69	...do	33	2.1 : 1
3	54	...do	21	2.5 : 1
4	41	...do	18	2.3 : 1
5	60	...do	19	3.1 : 1

PRECIPITATION AND IRRIGATION DURING GROWING SEASON IN AREAS STUDIED

The growing season for Montana forage crops might be said to commence in April. However, not much growth is apparent until May. During May and June the forage plants make their greatest growth, and the maximum assimilation of mineral plant food takes place during this period. By July the forage is usually fully matured.

All native and wild grasses collected during this study of mineral deficiencies represent nonirrigated vegetation. The alfalfa, clover, and timothy samples were collected from irrigated lands.

In the areas where samples of alfalfa and clover were gathered the first crop of hay is generally harvested the latter part of June or early in July. Timothy is usually cut at a date somewhat later. The second crop of alfalfa is invariably cut from six to seven weeks after the first crop.

The amount of irrigation water thought to be needed for the production of satisfactory crops varies in different regions depending upon the amount of precipitation. It also depends materially upon the nature of the crop, its root habit, and the nature of the soil. The amount of soil moisture most favorable to plant growth is about half the maximum it can hold. No record was available as to the amount of irrigation water that was used in the different localities where forage samples were collected, but from field observations made at the time of collecting the samples the irrigation was carried out in accordance with good average farm practice.

Table 9 gives the precipitation in three different areas for the years during which the investigation was made. The analyses of samples listed in Table 1, fall within the Bozeman area. In 1925 the average monthly precipitation for April, May, and June was 2.03 inches, as compared with 2.82 inches for the same period in 1927. The analyses of bunch-grass samples listed in Table 1, taken from nonirrigated land, and which are directly affected by precipitation, show a higher calcium and phosphorus content in 1925 than in 1927. Redtop samples from nonirrigated land (Table 1) show lower calcium and higher phosphorus content in 1925 than in 1927.

TABLE 9.—*Precipitation during the growing season (April to September, inclusive), for three districts in the survey of affected areas in Montana, 1925–1927*

District	Year	Precipitation in inches during—			3-month average	Precipitation in inches during—			Total for growing season	6-month average
		April	May	June		July	August	September		
Bozeman	1925	2.54	1.26	2.30	2.03	2.22	1.30	3.08	12.70	2.12
Do	1926	.35	4.82	1.59	2.25	.94	1.98	3.14	12.82	2.14
Do	1927	1.93	5.06	1.46	2.82	2.53	2.29	1.12	14.39	2.40
Three Forks	1925	0	1.20	3.33	1.51	.59	.78	1.79	7.69	1.28
Do	1926	.32	3.62	2.08	2.01	3.63	2.32	2.48	14.45	2.41
Do	1927	.91	4.10	1.69	2.23	.97	1.83	.70	10.20	1.70
Choteau	1925	2.33	.10	3.90	2.11	.57	1.38	2.37	10.65	1.77
Do	1926	.24	.65	4.55	1.81	.77	.36	3.15	9.72	1.62
Do	1927	.99	8.28	2.85	4.04	2.69	2.68	.62	18.11	3.02

The forage samples from irrigated lands listed in Table 1 all show higher lime and lower phosphorus content in the wetter season of 1927. As stated before, no trouble with bone-chewing cattle has been reported from the areas where these samples were taken. These data are not exactly in agreement with the findings of Forbes (*5, p. 94*) in irrigation experiments conducted at Fallon, Nev., and Yuma, Ariz., which show that the percentages of phosphorus in alfalfa, bluejoint, Indian bunch grass, and Bermuda grass was increased by increasing the moisture content of the soil during plant growth and that the percentage of calcium is lowered by an increase in moisture during plant growth.

The Three Forks data show an average monthly precipitation of 2.01 inches during April, May, and June for 1926 and an average of 2.23 inches monthly for the same period of 1927. The total precipitation and the average precipitation per month shows that 1926 was decidedly wetter than 1927 for the period from April to September inclusive, although the precipitation for the first three growing months was higher in 1927. The analyses listed in Table 4 fall within this Three Forks area. The phosphorus content of the wild-grass samples (Table 4) is less in 1927 than in 1926, even though the precipitation for the three months, when precipitation would be a factor, was higher. The calcium content of the wild-grass samples in this area was somewhat higher in 1926 than in 1927. The phosphorus content of alfalfa and clover (Table 4) is lower in samples collected during the growing season of 1927 than in those collected in 1926. The corresponding calcium content of these samples is higher in 1926 than in 1927.

The Choteau data show a higher average precipitation during the growing season of 1927 than they do for the growing season of 1926;

3.02 and 1.62 inches per month, respectively. Starting in May with a heavy precipitation of 8.28 inches, and following the next three months with 2.85, 2.69, and 2.68 inches, respectively, the entire growing season of 1927 might be considered as an especially wet one for areas in this territory. An inspection of Table 4 shows that there was a decided decrease in the phosphorus content of the wild-grass samples collected in 1927 compared with those collected in the dryer year of 1926. The calcium content of the samples remained about the same in both years, though there was a slight tendency toward a somewhat higher lime content in 1927. It must be remembered that the forage samples collected in these areas were grown on wet, swampy, nonirrigated soil. These wet meadows, as they might be called, are characterized by totally different flora than is found at higher elevations on the benches.

Wet conditions, such as were found in many of the pastures in the affected areas, are responsible for the killing out of many of the better grasses, and their replacement with rushes, sedges, and the less valuable forage plants. It should be borne in mind that the most valuable grasses, from the standpoint of feeding value, do not grow in swamps. Most of them are easily drowned out and replaced by others less valuable. Increased precipitation in these areas, already wet, might easily be assumed to decrease the calcium and phosphorus content of these grasses.

INTERPRETATION AND DISCUSSION

AMOUNTS OF CALCIUM AND PHOSPHORUS REQUIRED

The amounts of calcium and phosphorus that forage should contain to furnish a sufficiency of these elements for normal growth and development of cattle appears to be a matter of speculation. No definite standard seems to have been adopted by the various authorities on cattle feeding; i. e., no sharp dividing line seems to have been drawn between those forage feeds containing a deficiency of calcium and phosphorus and those containing adequate quantities. Hart and coworkers (*10*) say:

> The comparatively large amounts of calcium and phosphorus in our roughages, together with the large amounts of these feeds consumed by the horse and by ruminants, lessens the danger of a deficiency of these materials in their rations during growth. The margin of safety, however, can not be very wide, as in certain regions plants grown on soils low in calcium have been known to cause bone diseases which are ordinarily associated with an insufficient calcium supply.

In like manner soils low in phosphorus would have a depressing effect upon the phosphorus content of plants. At present there are few data available as to the minimum amounts of calcium and phosphorus which will permit the normal development of growing animals. Henry and Morrison (*12, p. 572*) give 1.95, 1.60, and 0.25 per cent, respectively, as the average CaO content of alfalfa, red clover, and timothy hay. For the same hays they give 0.54, 0.39, and 0.31 per cent, respectively, as the average P_2O_5 content. An inspection of the results obtained (Table 6) will show that the CaO content of alfalfa, both from unaffected and affected areas in this summary is higher than the Henry and Morrison figures. The CaO content of clover samples collected in affected areas is considerably higher (Table 6) than the figure given by Henry and Morrison. The calcium content of the wild grasses, both from affected and un-

affected areas is not high compared to that of legumes. However, the CaO content of these grasses (Table 6) is higher than that of timothy and bunch grass (Table 1) collected from areas where no mineral deficiencies have developed.

According to Eckles (*4*) the actual phosphorus requirement of cattle is as yet unknown, but it is not improbable that the optimum is close to that of other animals. Eckles states that McCollum considers 0.95 per cent phosphoric acid in the ration, on the dry basis, as an optimum for the well-being of laboratory animals. The phosphorus content of all forage samples collected in these investigations of mineral deficiencies is well under the McCollum optimum; this is especially true of the wild-grass forage.

COMPARISONS OF CALCIUM AND PHOSPHORUS CONTENT OF FORAGE GRASSES

When the amounts of calcium and phosphorus found in hay examined by other investigators in connection with mineral-deficiency studies in other localities (Table 10) are compared with the amounts found in Montana forage crops (Table 6) it may readily be seen that some of the forage in this State averages lower in phosphorus than that from other places. Nine samples of alfalfa hay, averaging 0.249 per cent P_2O_5 (Table 2) and six samples of freshly cut alfalfa, averaging 0.369 per cent P_2O_5 (Table 3) from an affected area in Madison County are both much below the average of 14 samples of alfalfa hay as reported by Eckles from affected farms in Minnesota. On the other hand, comparable Montana alfalfa is decidedly higher in calcium than those samples from affected farms in Minnesota. Ten samples of timothy hay, averaging 0.254 per cent P_2O_5 collected in 1927, in Gallatin County (Table 1) from an unaffected area, are only slightly higher in phosphorus than timothy samples collected from affected farms in Minnesota. Alfalfa hay from affected areas in Wisconsin averages in phosphorus content about the same as Montana alfalfa.

TABLE 10.—*Calcium and phosphorus content of hay in samples collected by various investigators of mineral-deficiency disorders in cattle*

Location, investigator, and crop	Samples	CaO	P_2O_5
	Number	*Per cent*	*Per cent*
Minnesota (Eckles):[1]			
Affected farms, prairie hay	30	0.578	0.230
Affected farms, timothy hay	4	.550	.245
Affected farms, alfalfa hay	14	2.628	.447
South Africa (Theiler):			
Affected area, hay	6	.443	.212
Norway (Tuff):			
Badly affected area, hay	3	.250	.142
Unaffected area, hay	1	.880	.440
Germany (Stohmann):			
Affected farms, hay	1	.630	.210
Unaffected farms, hay	1	.900	.430
Germany (Kellner):			
Affected farms, hay	5	.829	.238
Good hay (Wolf)	1	.950	.430
Australia (Guthrie):			
Affected area, hay	4	.378	.274
Unaffected area, hay	2	.558	.414
Wisconsin, Door County (Hart):[1]			
Affected area, sweet clover			.267
Do			.200
Do			.208
Affected area, alfalfa hay			.328

[1] These analyses quoted from Bulletin 229, University of Minnesota (*4*) and Bulletin 389, University of Wisconsin (*8*).

Wild grasses found in Montana can not be compared directly with those listed from other places because the species growing locally are entirely different from those growing elsewhere on which mineral-composition data have been published. Guthrie (*7*) reports the composition of six samples of native grasses from affected and unaffected areas in Australia. Four of these samples from affected areas average 0.277 per cent phosphoric acid and two samples from unaffected areas average 0.56 per cent phosphoric acid. The corresponding lime content averages 0.382 per cent and 0.415 per cent, respectively. These samples were mixtures of a variety of grasses from a number of different districts. Fourteen samples of wild grasses from different affected areas in Montana averaged 0.259 per cent P_2O_5 and 0.441 per cent CaO, respectively.

The experimental data indicate that our local legumes are better supplied with lime than are those legumes from affected areas in many other localities where studies of mineral deficiency have been made. The same does not hold true of the phosphorus content, however. The native local grasses from affected areas average about the same in phosphorus and calcium content as do those from affected areas in other places.

Tuff (*20*) gives the following analysis of three specimens of hay mown from marshes and outlying pastures in a badly affected area in Norway:

	Sample 1	Sample 2	Sample 3
Lime (CaO)..... per cent..	0.185	0.279	0.287
Phosphoric acid (P_2O_5)..... do....	.155	.143	.129

Tuff states that these hays contain about one-third the normal content of calcium and phosphorus. The grass samples collected in Teton County (Table 5) are analogous to these hays found in Norway. In the osteomalacious districts in Norway where these marsh hays are found, just as in Montana, bone-chewing disorders appear constantly every year and at all seasons, independent of rainfall, if no special measures are taken against them.

AVAILABILITY OF PHOSPHORUS IN LIME SOILS

As stated before, all samples of soil collected in this study of mineral deficiencies show a high lime content (Table 7) and seem adequately supplied with phosphorus. However, the phosphorus content of the forage plants grown on these lime soils in affected areas indicates that the phosphorus in the soil is not readily available as a plant food.

Fraps (*6*), who has done considerable work with lime and phosphate fertilizers, says that the literature with respect to the action of lime on soil phosphates is contradictory. He states that this may be due to the character of the soil phosphates; the lime may exert a depressing effect when the phosphorus occurs in the form of tricalcium phosphate, but its action may be favorable when the phosphorus occurs in the form of iron or aluminum phosphates. Fraps further states that the quantity of phosphoric acid taken up by crops may be affected by the lime, vegetable matter, or other soil constituents. If such is the case, deviations from the average must be due on the one hand to a

deficiency of activating soil ingredients and on the other hand, to an adequacy. Thus two soils containing the same quantity of available phosphorus might supply different quantities of phosphoric acid to growing plants by reason of an excess of a deficiency in some other plant-food element contained by them.

Unpublished data by the writer show that the soils from the regions where mineral-deficiency disorders have appeared in Montana respond in a most striking manner to the application of phosphate fertilizers; the phosphorus content of the forage grown upon these soils is increased after the application, and the crop yield as well.

RECONNOISSANCE SOIL SURVEY OF MONTANA

Chemical analyses of the types composing the different soil series in the reconnoissance soil survey now being made in Montana indicate that many of these soils might be classified as "phosphorus deficient"; also from the number of acid soils found, many other soils are deficient in lime. Even though the land where the available plant foods are depleted in lime and phosphorus, as is indicated by the analyses of some of the soils in these series (results as yet unpublished), might be farmed for a few years without any apparent deficiency in the mineral supply of plant food, it is certain that the original fertility can not last long. It is only a question of time until the use of lime will be necessary to correct some of these acid soils, and the need of phosphate fertilizers will become imperative.

RELATIVE METABOLISM OF CALCIUM AND PHOSPHORUS

Forbes (*5 p. 213*) states:

> The less the available supply of any nutrient, compared with the demand for the same, the more definitely does it become the limiting factor in production, and the more closely does its metabolism measure that of the body as a whole. Any element, therefore, might become the limiting factor in metabolism; thus, on a ration which is practically iron-free, the total growth might vary directly with the iron content of the ration.

Continuing further he says:

> In view of their distribution and relationships in the tissues it might be supposed that nitrogen, sulphur, calcium, and phosphorus would ordinarily be found somewhat closely interdependent in metabolism; and since phosphorus, as a universal cell constituent, is concerned in all growth and production, it would be assumed that this element would be found quantitatively related especially to those other elements whose metabolism is dominant during the particular kind of growth or production under observation.

Kellner (*14, p. 287*) concludes that a calf 5 to 6 months old utilized from 42 to 54 per cent of the lime and from 46 to 65 per cent of the phosphorus in its feed. He further states that animals should have in their ordinary food about two to three times as much lime and phosphoric acid as they store in their bodies.

Kellner (*14, p. 293*) computes that the skeleton of a year-old calf contains on an average 7,700 grams (17 pounds) CaO and 7,000 grams (15.5 pounds) P_2O_5. To produce this amount of these minerals a daily accretion of 21 grams CaO and 19 grams P_2O_5 would be required during the first year. The daily ration must therefore contain 40 to 60 grams (1.5 to 2 ounces) of each of these materials to supply these demands adequately. Assuming the average to be 50 grams, the amount of hay a calf would have to eat daily to secure the necessary calcium and phosphorus may be calculated.

The results of such a calculation based on the percentages of calcium and phosphorus given in Table 5, and using this wild grass as an example, are shown in Table 11. If Kellner's statement as to the quantities of calcium and phosphorus necessary to furnish the required amounts of these mineral substances be true, it can be seen that it would be quite impossible for a growing young calf to eat and digest enough of these hays to supply the necessary bodily requirements.

TABLE 11.—*Amounts of wild-grass hay necessary to furnish required daily amounts of calcium and phosphorus for growing calf*

[Calculated from 1926 data, Table 5]

Type of ranch	Per cent calcium as CaO in hay	Pounds of hay required daily in terms of CaO	Per cent phosphorus as P_2O_5 in hay	Pounds of hay required daily in terms of P_2O_5
Wet lowland	0.31	35.5	0.237	46.5
Do	.70	15.7	.133	82.8
Do	.53	20.8	.184	59.8
At higher elevation	.31	35.5	.275	40.0
Do	.36	30.6	.282	39.1

CALCIUM SUPPLY IN WATER

In considering the amounts of calcium and phosphorus derived by range cattle from their feed, the water consumed by them daily must be estimated. Table 12 contains figures of the amounts of water consumed daily by range cattle at different periods during growth. The figures are estimates and have been calculated to meet Montana conditions.

TABLE 12.—*Amounts of water consumed daily by Montana range cattle at different periods during growth*

Period of growth	Season	Pounds of water consumed daily
Aged steer	Summer	100–125
2-year-old steer	do	80– 90
Yearling steer	do	50– 60
Calf	do	[1] 40– 50
Dry cow	Winter	80
2-year-old heifer	do	80
Calf	do	60

[1] Water in milk additional.

Taking 70 pounds of water as the average amount used daily, summer and winter, by a yearling steer the calcium in the drinking water used may be calculated. For example, sample No. 5, Table 8, analyzed 60 parts per million Ca (84 parts per million CaO). Assuming that all of the lime in this water was assimilated, it would furnish for bodily requirements, 2.67 grams of CaO per day. This amount would help materially in making up any deficiency of lime in the feed. On the other hand, any deficiency of phosphorus in the feed could not be made up in the drinking water, because only a mere trace of phosphorus was found in any of the water samples analyzed.

PROPORTION OF CALCIUM TO PHOSPHORUS IN FORAGE

The proportion of calcium to phosphorus in the food supply is an additional factor in regulating the amount of phosphorus retained by the growing young animal. Hart and associates (*9*) say that the rate of growth will, in a large measure, control the demand for phosphorus and calcium, causing a variation in the amounts retained and that for bone formation a proper proportion of calcium for ultimate formation of calcium phosphate must accompany the phosphorus intake.

The proportion of phosphorus to calcium in tricalcium phosphate is as 1:1.9. By calculating the lime and phosphorus content of different forage samples (Table 6) as if combined to form tricalcium phosphate, the results (Table 13) show that the leguminous plants have a large amount of CaO in excess of the lime required for chemical combination with the phosphorus. This holds for the forage from both unaffected and affected areas. On the same basis the wild grasses show a slight excess of P_2O_5 in samples from the unaffected areas, but the corresponding samples from affected areas show an excess of CaO. These calculations are made to show that there is more possibility of the phosphorus than of the calcium content of forage grasses from affected areas falling short of a combining proportion. The legumes would naturally always contain a large excess of lime in comparison with their phosphorus content. Even though the wild-grass samples from unaffected areas had an excess of phosphorus, over and above the amount required to combine with the lime contained by these samples, it was only in slight excess.

TABLE 13.—*The percentages of tricalcium phosphate formed by combining calcium and phosphorus in different proportions and resultant excess as of CaO and P_2O_5*

[Calculated from percentages of CaO and P_2O_5 in forage plants shown in Table 6]

Calcium content of hay as CaO	Phosphorus content of hay as P_2O_5	Resultant $Ca_3(PO_4)_2$	Excess CaO	Excess P_2O_5
Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*
2.02	0.423	0.924	1.519	----------
2.59	.284	.620	2.254	----------
1.47	.356	.777	1.049	----------
2.27	.299	.655	1.916	----------
0.341	.360	.629	----------	0.072
.441	.259	.566	.134	----------

MAGNESIUM IN SOIL

Magnesium is not detrimental to optimum plant growth when it occurs in the soil as the natural carbonate, as in limestones and dolomites. Plants will tolerate larger amounts of an essential element than they require. The quantity of magnesium, as well as the quantity of calcium, taken up by plants is dependent upon the amount available and upon the kind of plants. Wyatt (*22*) in his studies on the influence of calcium and magnesium on plant growth, concluded that alfalfa grew normally either in 96 per cent of dolomite and 4 per cent of sand, 100 per cent of magnesian limestone, or in sand containing 7 per cent of magnesite, and that dolomite up to 40

per cent proved beneficial. From the analyses of soil samples taken in affected areas in Montana (Table 7) it may be observed that the percentage of magnesium in all samples falls within the range given by Wyatt. There is nothing in these analyses to indicate that the magnesium contained in the soil or forage samples from affected areas would produce any unusual conditions in the study of mineral deficiencies.

EFFECT OF PRECIPITATION

It is reasonable to assume that in areas where the soil is poor in lime and phosphoric acid a prolonged drought might easily cause osteomalacia the following year. Plants would naturally become poorer in mineral matter during a period of drought because the minerals would not be in solution in the dry earth. Precipitation would be a factor in the growth of forage plants from nonirrigated areas, whereas, forage plants from irrigated land would be affected slightly, if at all. No prolonged periods of drought were encountered, however, during this investigation. As a matter of fact, during the growing seasons mentioned in this report, the precipitation was above normal in the affected areas mentioned.

The analyses of grasses which make up the forage crops in Teton County (Table 5), from nonirrigated land suggest that the low content of phosphoric acid and lime in these grasses was the main source of trouble, occurring as an enzootic, independent of the amount of precipitation.

INVESTIGATIONAL RESULTS AND SUGGESTIONS

In the literature reviewed no cases of osteomalocia are given in which the lack of calcium alone has proved to be the only source of trouble. Aston (*23*) and Reakes (*16*, *17*) concluded that the severe trouble in New Zealand was due to the low phosphorus and iron content of the soil and vegetation. Tuff (*20*) in discussing osteomalacia in Norway concluded that the trouble there might be attributed to both calcium and phosphorus deficiency in the soil and forage. Theiler (*19*) in his extensive feeding experiments showed that deficiency trouble with cattle in the South African veldt was due to a shortage of phosphorus in the forage grasses of osteomalacious districts. König and Karst (*15*) were inclined to attribute mineral deficiency trouble in Germany to the alkaline reaction of hay ash and the excess of sulphates. Henry (*11*) and Guthrie (*7*) seem convinced that the low phosphorus feeds grown in affected areas were responsible for mineral deficiency troubles in Australia. Eckles (*4*) concluded that mineral deficiency among cattle in Minnesota is caused chiefly by the lack of phosphorus, rather than calcium, in the roughages fed, and has proved by extensive feeding experiments that phosphorus was the deficient element. Hart (*8*) also reports that the nutritional disorder of cattle in Wisconsin might be attributed to mineral deficiency in forage-crop feeds, and especially to a lack of phosphorus.

From the data which have been given it is evident that many of the Montana grasses are lacking in phosphorus, rather than lime. These data are also in agreement with those supplied by a number of other investigations previously mentioned herein. However, final conclusions can not be reached in a study of this kind on a basis

of chemical findings alone, and, furthermore there are other affected areas in the State in which conditions have not been studied. It is quite possible that other factors might become apparent if the scope of this investigation was extended.

In spite of prolonged investigations the etiology of osteomalacia is not yet fully understood. The results of mineral metabolism investigations carried on in recent years show that the proportion between acid and basic equivalents in the total foodstuffs, and the proportion between the various metalic ions in the inorganic salts in the food are of very great importance in regulating the normal metabolism of minerals.

The fact should be reckoned with that the majority of cases of osteomalacia are caused mainly by an insufficiency of mineral elements in the foodstuffs.

Another matter of importance is the fact that the ranges in many parts of Montana already show signs of exhaustion and the number of stock supported upon a given area is steadily diminishing. The tendency of stockmen to purchase or lease these ranges for private use tends to make the question of their improvement increasingly important.

The prevention of osteomalacia is more important than its cure. Although it would not be practical in every case to manure pastures with the mineral substances in which they are deficient, the seeding of various species of grasses found most hardy under pasturage and most drought resistant in dry seasons, and the determination of their relative forage value, are worthy of consideration.

SUMMARY AND CONCLUSIONS

Cattle have long been known to display abnormal or perverted appetites and other unusual disorders when fed rations deficient in minerals. Many Montana forage crops, while plentifully supplied with calcium, are deficient in phosphorus. This phosphorus deficiency results in a calcium excess when the forage is fed to animals and this is physiologically detrimental to cattle, giving rise to various mineral-deficiency disorders. Calcium, however, is not deficient in forage crops grown in Montana localities where such mineral-deficiency disorders are common among cattle, and herein called "affected" areas.

All forage-crop samples from such affected areas show a lower phosphorus content than corresponding forage crop samples from unaffected areas. The same does not hold for calcium and magnesium. Some of the grasses and forage crops from affected areas in Montana are lower in phosphorus content, but tend to contain more calcium than similar plants from affected areas elsewhere.

The analyses of the soil samples collected in affected areas show a high calcium content, while the phosphorus content is apparently adequate. Since the forage crops grown on these soils are phosphorus deficient it is to be inferred that the soil phosphorus is for some reason not readily available for plant assimilation.

The lime supply for the bodily requirements of growing cattle studied in this investigation was augmented by the calcium contained in their drinking water.

Magnesium does not appear to have any significant effect in causing the deficiency disorders studied.

The amount of precipitation in various regions during the different years the investigations were carried on appears to have had no effect upon the mineral content of the grasses and forage crops and also to have been negligible in its effect upon other aspects of this investigation. Wet meadows appear to be responsible for the poor quality of grasses from the standpoint of their value as feeds for cattle, the better grasses being killed off and replaced by rushes, sedges, and other less valuable forage plants.

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